Glyc-*O*-genetics of Walker-Warburg syndrome

PhD thesis: **Glyc-*O*-genetics of Walker-Warburg syndrome**
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Glyc-*O*-genetics of Walker-Warburg syndrome

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Chapter 1

Introduction and outline of this thesis

1.1 General introduction

Walker-Warburg syndrome (WWS) is the most severe clinical entity in a group of disorders that show clinical overlap and share a common pathomechanism. At birth, patients show hydrocephalus and other severe defects of the central nervous system, eye abnormalities, and profound muscle weakness. Brain imaging reveals a number of severe structural malformations, including disruption of the cortical layers and absence of gyri (lissencephaly), absence of the corpus callosum, thin brainstem and a small cerebellum and sometimes encephalocele. Most of these brain malformations are indicative of neuronal migration defects. Elevated creatine kinase (CK) levels in the blood point to muscular dystrophy. Most WWS patients die in their first year of life due to complications such as respiratory infection. The common pathomechanism for WWS and related disorders is a defect in the *O*-linked glycosylation of α-dystroglycan. Specific *O*-mannosyl glycans are crucial for the function of dystroglycan and hypoglycosylation results in a loss of binding of extracellular ligands, such as laminin-α2. Prior to the work described in this thesis, recessive mutations were reported in three genes, *FKRP*, *FKTN*, and *POMT1*, in approximately one-fifth of the WWS patients in our research cohort (reviewed in Chapter 2).

The research described in this thesis is focused on the elucidation of other genetic factors involved in WWS. Elucidation of these factors is urgently needed to improve genetic counseling and DNA-diagnostics for families confronted with this devastating syndrome. The identification of causative mutations in new genes may also help to unravel the molecular mechanisms of disease for clinically overlapping disorders. Finally it provides more insights in the biological processes that are required for neuronal migration in brain and eye development, and for muscle integrity.

The next paragraphs of this chapter provide background information on protein glycosylation in health and disease, and the approaches taken to WWS disease gene identification.

1.2 Protein glycosylation

All cells of every species studied so far are covered by carbohydrate structures, referred to as glycans (Varki, 2007). Glycans are sugar chains, synthesized and covalently linked to proteins and lipids in a process called glycosylation. Well-known glycans are those found on blood cells, defining the blood groups A, B, AB, and O (Landsteiner, 1931; Yamamoto et al., 1990). Cell-surface glycans were first

discovered on rat cells by electron microscopy (Rambourg and Leblond, 1967). Until two decades ago, it was assumed that glycans are only found facing away from the cytosol on the plasma membrane or on membranes of intracellular organelles or on secreted molecules (Varki, 2006). Glycans are remarkably diverse in structure, and function in a broad range of biological processes. They mediate signalling, cell-cell and host-pathogen interactions, and trafficking of glycoconjugates. In addition, glycans have intrinsic functions like modification of protein properties such as solubility and stability, and are structurally involved in cell membranes and the extracellular matrix (Sharon, 1996; Taylor and Drickamer, 2006).

Glycosylation mainly occurs in the endoplasmic reticulum (ER) and Golgi compartments, also referred to as the secretory pathway. An exception to this are single *O*-linked *N*-acetylglucosamine modifications of proteins in cytosolic and nuclear compartments that have regulatory functions, often competing with other protein modifications such as phosphorylation (Hart et al., 2007). Protein glycosylation occurs at a nitrogen atom (*N*-linked) of an asparagine residue, or at an oxygen atom (*O*-linked) of serine and threonine residues (Schachter, 2000; Yan and Lennarz, 2005). Less common types of glycosylation of specific proteins occurs on lysine, tyrosine, and tryptophan residues (Ohtsubo and Marth, 2006). *N*-linked glycosylation is a highly ordered process characterized by a standard repertoire of sequential additions and removals of sugar groups. The *N*-linked glycosylation pathway has been resolved in great detail and can be divided into three stages: 1) formation of a lipid-linked precursor oligosaccharide; 2) *en bloc* transfer of the oligosaccharide to the polypeptide; 3) processing of the oligosaccharide. The first two steps and initial trimming reactions (removal of some of the original sugar residues) occur in the ER. Further processing takes place as the nascent glycoprotein migrates through the Golgi apparatus (Marquardt and Denecke, 2003). *N*-linked glycans have a common protein-glycan linkage and core structure, resulting in a relative limited number of different glycan structures. In contrast, *O*-linked glycosylation produces many different glycans structures with eight different protein-glycan linkages (**Figure 1**).

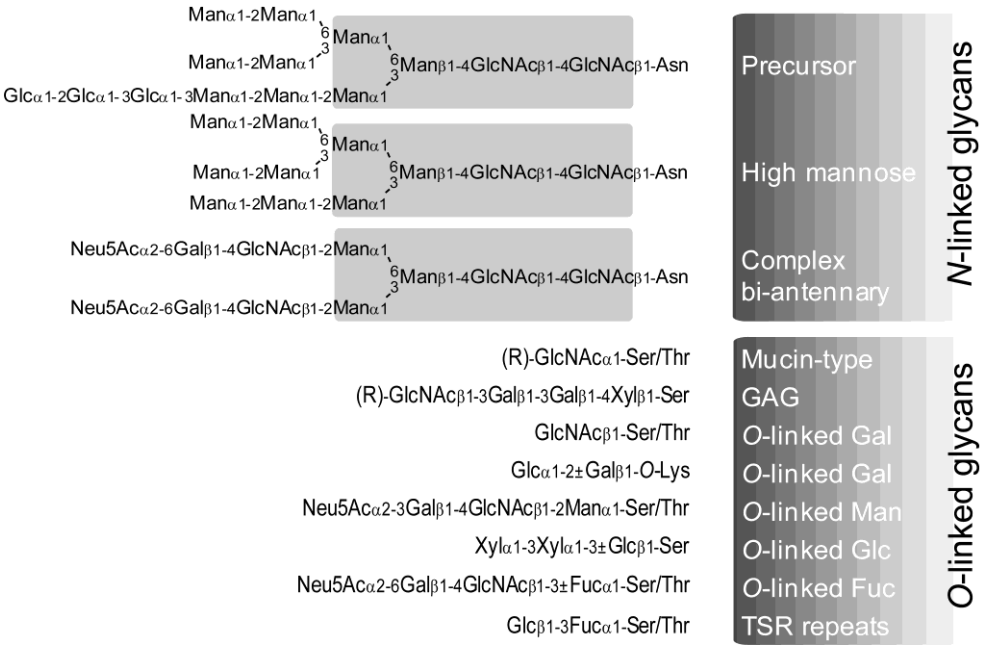

Figure 1. Different types of *N*-, and *O*-linked glycosylation. *N*-linked glycans are attached to an asparagine residue and have a common core structure (shaded), resulting in a relatively limited number of different *N*-glycan structures. *O*-linked glycans are attached to serine, threonine or lysine residues. Residues at the reducing end of *O*-glycans (R) are often modified, resulting in many different glycan structures.

Glycosyltransferases in *O*-linked glycosylation are analogous to those in the *N*-linked biosynthetic pathway, however, there is no precursor oligosaccharide nor *en bloc* transfer of oligosaccharides onto the protein substrate. Glycans are added as monosaccharides from nucleotide sugar donors mainly in the Golgi compartments, which has parallels with the terminal modification during processing of *N*-linked glycans. Another difference is the apparent lack of a consensus target sequence for *O*-linked glycosylation analogous to the Asn-Xaa-Ser/Thr sequences that define *N*-linked glycosylation sites. Numerous glycosyltransferases can attach GalNAc to serine and threonine residues, however, they have specificity for different amino acid sequences surrounding the glycosylation target (Taylor and Drickamer, 2006). Although glycosaminoglycans (GAGs, or proteoglycans) are also *O*-linked, these glycans are linear, and often highly sulfated and produced by different biosynthetic pathways (Esko and Selleck, 2002). The importance of glycosylation in post-translational protein modification and lipid metabolism is reflected by the involvement of a high number of genes, approximately 1% of the mammalian transcriptome, that encode glycosylation functions (Lowe and Marth, 2003). Unlike

DNA, RNA, and protein synthesis, the biosyntheses of glycans is not template driven and subject to multiple sequential and competitive enzymatic pathways (Esko and Selleck, 2002; Kornfeld and Kornfeld, 1985; Maccioni et al., 2002; Schachter, 2000). The array of glycan structures present on a surface of a specific cell depends on many factors involved in cellular regulation of glycan expression, and therefore is difficult to predict. Glycan expression is regulated by multiple mechanisms that alter the expression and activity of glycosyltransferases and glycosidases, which catalyze the synthesis and hydrolysis of glycan structures, respectively. These mechanisms include glycosyltransferase and glycosidase gene transcription, synthesis and transport of nucleotide sugar donors to the ER and Golgi, modulation of enzymatic activity through phosphorylation, substrate competition, intracellular enzyme trafficking, and glycan turnover at the cell surface by endocytosis (Ohtsubo and Marth, 2006).

Given the complexity of processes involved in glycosylation and the participation of glycans in many key biological processes, it is not surprising that genetic defects in glycan biosynthesis underlie the cellular mechanisms of many diseases.

1.3 Glycosylation defects and disease

In the past decade, about 40 genetic defects in the glycosylation pathways have been reported in the literature that result in a broad range of clinical phenotypes affecting nearly all organ systems. Most glycosylation disorders known to date affect *N*- or *O*-linked protein glycosylation. Almost invariably, genetic glycosylation disorders follow a recessive inheritance pattern, indicating that the remaining normal gene product in heterozygotes is sufficient to sustain normal physiological processes. Glycosylation in cellular mechanisms of health and disease was recently comprehensively reviewed (Freeze, 2006; Ohtsubo and Marth, 2006; Wopereis et al., 2006).

In general, genetic disorders that affect the *N*-linked glycosylation pathway are referred to as congenital disorders of glycosylation (CDG). Over 20 different autosomal recessive CDGs with a known genetic defect have been reported (Foulquier et al., 2007; Freeze, 2006; Kranz et al., 2007a; Kranz et al., 2007b). The CDGs are grouped in CDG type I and type II, and subtypes that are caused by mutations in different genes are indicated by lower case letters; CDG-Ia – CDG-Im, and CDG-IIa – CDG-IIh. Type I CDGs are caused by defects in the synthesis of the lipid-linked oligosaccharide precursor in the endoplasmic reticulum, which results in inefficient and/or incomplete transfer of the oligosaccharide by an oligosaccharyltransferase to an asparagine residue. Defects in the further processing

of the protein *N*-linked glycans originate in the Golgi and are referred to as CDG type II.

With exception of CDG-Ia, which is caused by mutations in the *PMM2* gene, only few patients have been identified for each CDG subtype. The limited number of patients and overlapping clinical features make it difficult to clinically define CDG subtypes. In general, CDG patients present with neurological and multi-systemic diseases, with common symptoms such as psychomotor retardation, seizures, central nervous system abnormalities, hypotonia, coagulation defects, and liver disease. More specific clinical signs for CDG-I, such as inverted nipples, abnormal fat distribution, and hypoplasia of the cerebellum, may guide an early diagnosis. CDG-II patients are diagnosed with "classical" CDG features in combination with other unique features such as haemorrhages, cutis laxa, and impaired immune system (Wopereis, 2006). Unsolved cases of CDG-II (CDG-IIx) present specific clinical symptoms such as deafness, blindness, and urogenital anomalies (Wopereis et al., 2005). The diagnosis of CDGs is guided by biochemical analysis of glycans. Plasma transferrin is a sensitive and commonly used marker that can be investigated by various methods including isoelectric focusing, high-performance liquid chromatography, capillary electrophoresis, and mass spectrometry. These techniques allow for the detection of altered glycosylation and help to focus the search for the defect, however they cannot pinpoint the defect. Specific biochemical assays are developed to analyze i.e. enzyme activities and glycosylation intermediates. Particularly the first steps in *N*-linked glycosylation are highly conserved in evolution and comparison of lipid-linked oligosaccharide structures of yeast mutant strains with those of CDG type I patients resulted in the elucidation of genetic defect of various CDG type I disorders (Wopereis et al., 2005). Only few mouse models exist for human CDG genes. Null-alleles for most mouse orthologs of the CDG genes are not compatible with life in mice. Likewise, human null alleles are also not compatible with life as most patients with a CDG carry hypomorphic alleles rather than complete loss-of-function alleles (Freeze, 2006).

An emerging new group of CDG-II defects is characterized by defects in trafficking of multiple glycosyltransferases and in nucleotide-sugar transporters, which affect multiple glycosylation pathways. CDG-IIe is the first disorder where mutations in a subunit of the conserved oligomeric Golgi (COG) complex, COG7, were described that disrupt Golgi trafficking, resulting in abnormal *N*-, and *O*-linked glycosylation (Wu et al., 2004). Mutations in two other COG subunits were recently identified, COG1 in CDG-IIg, and COG8 in CDG-IIh (Foulquier et al., 2006; Foulquier et al., 2007; Kranz et al., 2007b). These findings indicate that disruption intra-Golgi trafficking of protein complexes may account for other types of glycosylation

disorders with combined defects in *N*- and *O*-linked protein glycosylation. This was demonstrated by the recent identification of loss-of-function mutations in the a2 subunit of the V-type H^+-ATPase in several families with syndromal cutis laxa and CDG-II, which also cause an impairment of Golgi trafficking probably due to disruption of local pH gradients (Kornak et al., 2008).

Most disorders caused by abnormal *O*-linked glycosylation are not included in the CDG nomenclature. They comprise a larger group of patients with a highly variable clinical presentation. Wopereis et al., comprehensively reviewed the *O*-glycan biosynthesis and the clinical and molecular aspects of defects in this process (Wopereis et al., 2006). The variable composition, linkage, and branching of *O*-glycans makes the detection of defects in *O*-glycan biosynthesis more complex than for *N*-glycan defects. Recently, an isoelectric focusing assay of a plasma protein, apolipoprotein C-III (ApoCIII), has been developed to screen for defects in biosynthesis of a mucin-type *O*-glycan found on many secreted proteins (Wopereis et al., 2003). However, most congenital defects of *O*-linked glycosylation have been identified by genetic studies. **Table 1** lists currently known disorders of *O*-linked glycosylation.

A distinct group of disorders is caused by defects in the synthesis of *O*-linked mannose glycans for which the only known target is α-dystroglycan. Defects in the synthesis of these glycans can be detected by immunohistochemical staining of α-dystroglycan on muscle biopsies with monoclonal antibodies VIA4-1 and IIH6 (Michele et al., 2002). It is not clear which carbohydrate epitope is recognized by the antibodies, and abnormal staining may occur also for defects in *N*-glycan or mucin-type *O*-glycan biosynthesis (Huizing et al., 2004; Wopereis et al., 2006). Defects in the *O*-linked mannose glycosylation of α-dystroglycan cause a disease spectrum of congenital muscular dystrophy (CMD) syndromes (**Table 1**), including structural brain and eye abnormalities in severe cases. These CMDs are denoted merosin-positive, because patients have no immunohistochemical abnormalities in laminin-α2 (merosin), which is seen in common forms of CMD, the laminopathies. It recently became evident that different mutations in all known genes involved in these disorders give rise to clinical variability, and to an overlap in clinical features of these disorders. Chapter 2 reviews the clinical characteristics, molecular genetics, and disease mechanisms of the most severe disorders in this clinical spectrum, Walker-Warburg syndrome and related disorders, as known in mid-2004. Disorders with disrupted *O*-mannose glycosylation are extremely rare and show a pattern of recessive inheritance. The incidence of these disorders, as for other rare autosomal recessive conditions, is higher in populations with a high rate of consanguineous unions. This phenomenon may be exploited to identify candidate disease loci.

Table 1. Human disorders of *O*-linked glycosylation

Disorder	OMIM	Gene
***O*-Gal**		
Ehlers-Danlos syndrome type VI	225400	*PLOD*
***O*-GalNAc**		
Familial Tumoral Calcinosis	211900	*GALNT3*
Tn syndrome	230430	*COSMC*
***O*-glycan sialylation**		
Congenital disorder of glycosylation type IIf	603585	*SLC35A1*
Distal myopathy with rimmed vacuoles	605820	*GNE*
Inclusion body myopathy 2	600737	*GNE*
Sialuria	269921	*GNE*
***O*-Man**		
Congenital muscular dystrophy, no MR	607155	*FKRP, FKTN*
Congenital muscular dystrophy + MR	606612	*FKRP, POMT1*
Congenital muscular dystrophy + CRB	608840	*FKRP, LARGE, POMT1, POMT2*
Fukuyama congenital muscular dystrophy	253800	*FKTN*
Limb-girdle muscular dystrophy+ MR	609308	*POMT1, POMT2*
Limb-girdle muscular dystrophy, no MR	607155	*FKRP, FKTN, POMGnT1, POMT2*
Muscle-eye-brain disease	253280	*POMGnT1*
Muscle-eye-brain disease like		*FKRP, POMT1, POMT2*
Walker-Warburg syndrome	236670	*POMT1, POMT2, FKTN, FKRP, LARGE*
***O*-Xyl**		
Multiple hereditary exotosis	133700, 133701, 600209	*EXT1, EXT2, EXT3*
Progeroid variant of Ehlers-Danlos syndrome	130070	*B4GALT7*
***O*-Xyl sulfation**		
Chondrodysplasias	600972, 222600, 256050	*DTDST*
Macular Corneal Dystrophy	217800	*CHST6*
Multiple Epiphyseal Dysplasia	226900	*DTDST*
Spondyloepiphyseal dysplasia	608637, 603005	*CHST3, ATPSK2*
***N*-, and *O*-glycan**		
Congenital disorder of glycosylation type IIc	266265	*FUCT1*
Congenital disorder of glycosylation type IId	607091	*B4GALT1*
Congenital disorder of glycosylation type IIe	608779	*COG7*
Congenital disorder of glycosylation type IIg	611209	*COG1*
Congenital disorder of glycosylation type IIh	611182	*COG8*
Cutis laxa, autosomal recessive, type II	219200	*ATP6V0A2*
Wrinkly skin syndrome	278250	*ATP6V0A2*
Anderson disease	607689	*SARA2*
Chylomicron retention disease	246700	*SARA2*
Chylomicron retention disease with Marinesco-Sjogren syndrome	607692	*SARA2*
Bombay blood group	211100	*FUT1, FUT2*
Para-Bombay blood group	211100	*FUT1*
Non-secretor blood group	182100	*FUT2*
Lewis-negative blood group	111100	*FUT3*

MR, mental retardation; CRB, cerebellar involvement

1.4 Disease-locus identification in autosomal recessive disorders

It was noted as early as 1902 that an unusually high number of patients with alkaptonuria were born to consanguineous parents (Garrod, 1902). The Mendelian explanation for this phenomenon was given soon after, and it was also predicted and observed that the rarer a disease the more pronounced this phenomenon (Lander and Botstein, 1987). A consanguineous marriage is most commonly defined as a union between a couple related as second cousins or closer. In theory, first-cousins share 1/8 of all their alleles, and seconds-cousins share 1/32 of all their alleles. Descendants of consanguineous parents inherit half of these common alleles (**Figure 2**). The assembly of two rare disease alleles is therefore more frequently observed in descendants from consanguineous parents compared to descendants from unrelated parents.

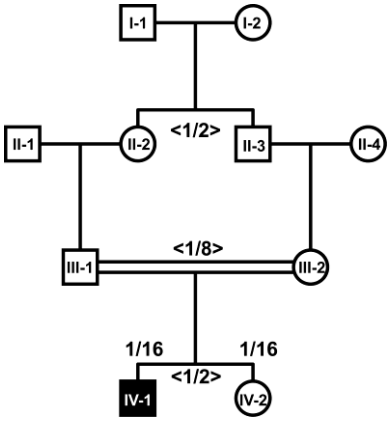

Figure 2. Example of a family pedigree showing a first-cousin relationship between individuals III-1 and III-2, represented by a double line (consanguinity). Half of the genome between individuals II-2 and II-3, and IV-1 and IV-2 is identical by descent. Individuals III-1 and III-2 share 1/8 of their genome. Half of their shared genome (1/16) is inherited by their children which is identical by descent (homozygous). Females are represented by circles, males by squares. Open symbols represent the unaffected family members, the solid black symbols the affected sibling.

In 1953, Cedric Smith noted the theoretical efficiency of studying inbred affected children, given a tightly linked highly polymorphic marker. The paucity of such highly polymorphic markers and the inability at that time to construct complete linkage maps of less polymorphic markers led Smith to conclude that "the method is impractical" (Smith, 1953). His theory relied on the fact that a child with a recessive disease with consanguineous parents co-inherits a homozygous (also referred to as autozygous) region of many centimorgans (cM) surrounding the disease locus. Depending on the degree of parental relationship, several regions of homozygosity will be present in any child from consanguineous parents, however, these will vary between children and only a homozygous disease locus will be "shared" by patients

with a genetically homogeneous disorder. Upon completion of a restriction fragment length polymorphism (RFLP) linkage map of the human genome in 1987, Eric Lander and David Botstein further explored the possibility to detect homozygosity by descent in order to map recessive traits in humans. The method should make it possible to map rare recessive diseases for which it is impractical or impossible to collect adequate numbers of families with multiple affected offspring. Based on their calculations, identification of a disease locus by homozygosity mapping should be feasible with few inbred patients when a human genetic map is available with a marker spacing of 10 cM, and with markers that are homozygous in at most half of the population (Lander and Botstein, 1987). The first successful applications of homozygosity mapping were in 1992, when the genetic defects were mapped for Werner syndrome to 8p, for familial Mediterranean fever to 16p, and Tunisian autosomal Duchenne-like muscular dystrophy to 13q (Ben Othmane et al., 1992; Goto et al., 1992; Pras et al., 1992). Homozygosity mapping is limited by the availability of affected offspring of related parents, however, geographical isolation and cultural norms resulted in populations where consanguinity is widespread. Contrary to what many people from western countries assume, consanguinity is not restricted to populations which are geographically or religiously isolated, instead, consanguineous marriages are favoured in many populations. In some populations the rate of consanguineous marriages exceeds 50% (**Figure 3**) (Bittles, 2001). Calculations predict that 6% (1/16) of the genome of a child of first cousins will be homozygous with on average homozygous segments of 20 cM. However, a study that quantified homozygosity in populations that have preferred consanguineous marriage for many generations showed that children of first-cousin parents have an overall homozygosity of approximately 11% (range 5-20, n=38), with on average a disease causing homozygous segment of 26 cM (range 5-70, n=100). This raise in overall homozygosity increases the chance of finding false linkage for a disease locus in small families, and shows that calculations based on pedigrees may underestimate the percentage of homozygosity (Woods et al., 2006).

Until four years ago, the standard method for identifying disease loci involved a whole-genome scan using a set of 300-400 highly informative multi-allelic microsatellite markers equally spaced across the genome. However, the enormous progress in detection and genotyping of single-nucleotide polymorphisms (SNPs) resulted in a growing popularity of using SNP markers. To date, more than 6.5 million human SNPs have been identified (validated SNPs, NCBI dbSNP Build 129) by large sequencing efforts like the HapMap project (http://www.hapmap.org). Despite a lower information content of the mostly biallelic SNP-markers (Kennedy et al., 2003), the rapid and highly automated genotyping of SNPs allows for high-throughput screening of nearly one million SNP-markers (Genome-wide SNP array

6.0, Affymetrix) at the time of writing this thesis. Several studies have compared whole-genome scan data from SNP-markers (>10K SNPs) with traditional 10-cM microsatellite marker set (~400 Simple Tandem Repeat markers), and showed the two major advantages of using the high-density SNP-scan over microsatellites; higher overall information content resulting in a better definition of linkage peaks, and speed of genotyping and downstream data processing (John et al., 2004; Middleton et al., 2004). In addition, these more dense arrays also allow for copy number detection by which small deletions and duplications can be mapped.

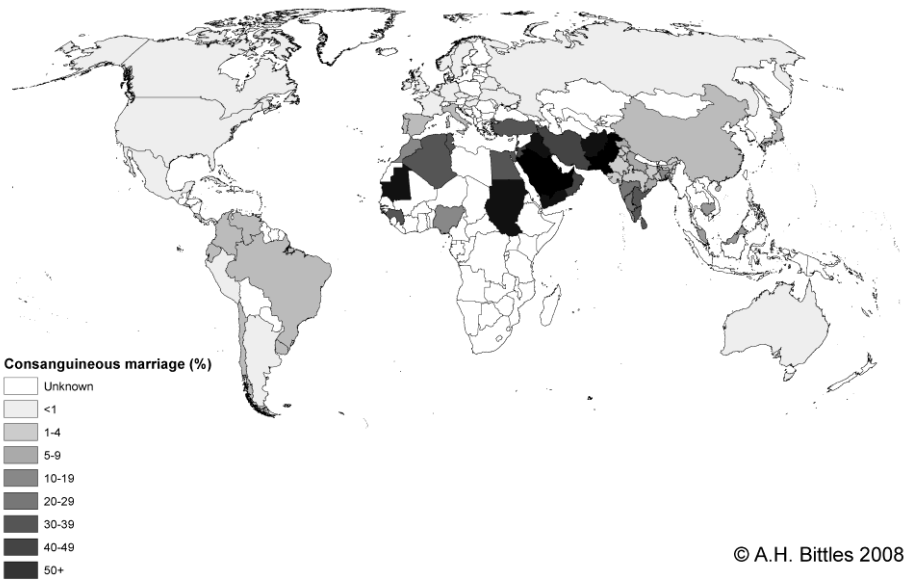

Figure 3. World-wide prevalence of consanguinity (www.consang.net, reprinted with permission of A.H. Bittles)

1.5 Positional candidate disease gene selection

For genetically heterogeneous and severe disorders, such as WWS, a candidate disease locus can be several megabases in size, often containing 50 or more genes. Comprehensive analysis of so many candidate genes is very time-consuming and expensive. Public availability of data derived from various genome-sequencing projects, and large-scale "omic" studies, resulted in a wealth of biological information, stored in many different databases (Kanehisa and Bork, 2003; Stein, 2003). To facilitate retrieval of such information from frequently used databases, retrieval systems have been developed, such as Entrez, MRS, and SRS (**Table 2**). Genome browsers, such as the University of California, Santa Cruz (UCSC) Genome Browser, and Ensembl (**Table 2**), integrated data from various sources with positional information making these browsers indispensable for users who study genes based on genomic context and in relation to their biological function or malfunction resulting in disease. Both, the UCSC, and Ensembl genome browsers provide a tool, Gene Sorter and BioMart, respectively, for step-by-step data filtering, based on genomic position, gene expression, gene ontology, and many other criteria. Many computational methods have been developed for bioinformatics analysis of a candidate disease locus to select the most likely candidate disease genes. Various tools have been developed to assist these researchers in systematic prioritization of candidate genes. **Table 2** lists a selection of tools, which are commonly used for candidate disease gene prediction or prioritization. Recent papers discuss the differences in methodology for different tools, and the application of a concert of methods to prioritize candidate genes (Oti and Brunner, 2007; Tiffin et al., 2006; van Driel and Brunner, 2006).

Table 2. Tools for retrieval of biological data, candidate disease gene prediction or prioritization

Name	Description	URL or reference
Data retrieval tools		
Entrez	NCBI's life sciences cross-database search tool	http://www.ncbi.nlm.nih.gov/Entrez
MRS	Cross-database search tool	http://mrs.cmbi.ru.nl
SRS	Cross-database search tool including data analysis tools	http://srs.bioinformatics.nl
Human genome browsers		
Ensembl	Annotated assembly of the complete human genome	http://www.ensembl.org/Homo_sapiens
UCSC Genome browser	Annotated assembly of the complete human genome	http://genome.ucsc.edu/cgi-bin/hgGateway?org=Human
Disease gene prediction		
Disease gene prediction (DGP)	Based on protein sequence properties and evolutionary conservation of known disease proteins	http://cgg.ebi.ac.uk/services/dgp
Protein–protein interactions	List of 300 candidate disease gene predictions based on protein-protein interactions	no web tool (Oti et al., 2006)
Gene prioritization		
Endeavour	Prioritization of positional candidate disease genes based on sequence, gene expression, functional annotation, pathways, and literature mining	http://www.esat.kuleuven.be/endeavour
Genes2Diseases (G2D)	Prioritization of positional candidate disease genes based on sequence, functional annotation, and literature mining	http://www.ogic.ca/projects/g2d_2
GeneSeeker	Filters positional candidate disease genes based on expression and phenotypic data from human and mouse	http://www.cmbi.ru.nl/GeneSeeker
Oti et al., 2008	Conserved co-expression for candidate disease gene prioritization	http://www.cmbi.ru.nl/~moti/coexpression/
POCUS	Selection of candidate disease genes based on shared functional annotation, InterPro domains, and expression profiles with two or more known disease genes	no web tool (Turner et al., 2003)
Prioritizer	Prioritization of positional candidate disease genes based on sequence, gene expression, functional annotation, pathways, and literature mining	http://www.prioritizer.nl
SUSPECTS	Prioritization of positional candidate disease genes based on gene and protein sequence properties, functional annotation, and gene expression	http://www.genetics.med.ed.ac.uk/suspects
Tiffen et al.	Prioritization of candidate disease genes based on literature mining and expression	no web tool (Tiffin et al., 2005)

1.6 Outline of this thesis

Work described in this thesis covers the elucidation of the genetic basis of WWS and related disorders. **Chapter 2** reviews the clinical characteristics, and genetics of WWS known at the start of my research project in 2004. **Chapters 3 and 4** describe the identification of two new WWS genes. Small gene mutations were identified in *POMT2* in four WWS patients from three unrelated families. Furthermore a 63-kb intragenic deletion in the *LARGE* gene was found in one WWS family. **Chapters 5 and 6** describe the phenotypic differences observed for different mutations in *POMT1* and *FKRP*. Seven new *POMT1* mutations were identified in patients with a less severe phenotype compared to WWS consisting of mental retardation, CMD and calf hypertrophy. In addition, seven new *POMT1* mutations are reported in WWS patients. A case reports describes a WWS family with homozygous *FKRP* startcodon mutations. A final discussion and prospects on the glyc-*O*-genetics of WWS and related disorders is given in **Chapter 7**.

Chapter 2

Glyco-*O*-genetics of Walker-Warburg syndrome

van Reeuwijk J, Brunner HG, van Bokhoven H.

Clinical Genetics 67: 281-289, 2005

Abstract

Walker-Warburg syndrome is the most severe of a group of multiple congenital anomaly disorders known as the cobblestone lissencephalies. These are characterized by congenital muscular dystrophy in conjunction with severe brain malformation and ocular abnormalities. In the last 3 years, important progress has been made towards the elucidation of the genetic causes of these disorders. Mutations in three genes, *POMT1*, *fukutin* and *FKRP*, have been described for Walker-Warburg syndrome, which together account for approximately 20% of patients with Walker-Warburg syndrome. It has become evident that some of the underlying genes may cause a broad spectrum of phenotypes, ranging from limb-girdle muscular dystrophy type 2I to Walker-Warburg syndrome. In some cases, a genotype-phenotype correlation can be recognized. In line with the known or proposed functions of the resolved genes, all patients with cobblestone lissencephaly show defects in the *O*-linked glycosylation of the glycoprotein α-dystroglycan. Perhaps, the missing genes underlying the remainder of the unexplained Walker-Warburg syndrome patients have also to be sought in the pathways involved in *O*-linked protein glycosylation.

Introduction

Walker-Warburg syndrome (WWS; MIM236670) was named after Walker, who reported the first patient with lissencephaly (smooth brain) in 1942 (Walker, 1942), and after Warburg who proposed its autosomal recessive inheritance (Warburg, 1976; Warburg, 1978). Patients with this rare developmental disorder have a life expectancy of less than 3 years (average 0.8 years). Numerous congenital anomalies have been reported, but the most consistent are malformations of three structures: muscle, eye and brain. This triad distribution of developmental defects is also found in muscle-eye-brain disease (MEB; MIM 253280) and Fukuyama congenital muscular dystrophy (FCMD; MIM 253800), which together with WWS comprise the group of the cobblestone lissencephalies. The clinical features of WWS are most severe, especially with regard to the brain phenotype, which may be diagnosed prenatally (Dobyns et al., 1989). Typical brain anomalies include hydrocephalus, neuronal overmigration, causing a cobblestone cortex, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, dilatation of the fourth ventricle, cerebellar hypoplasia and occasionally occipital encephalocele (**Figure 1**) (Cormand et al., 2001).

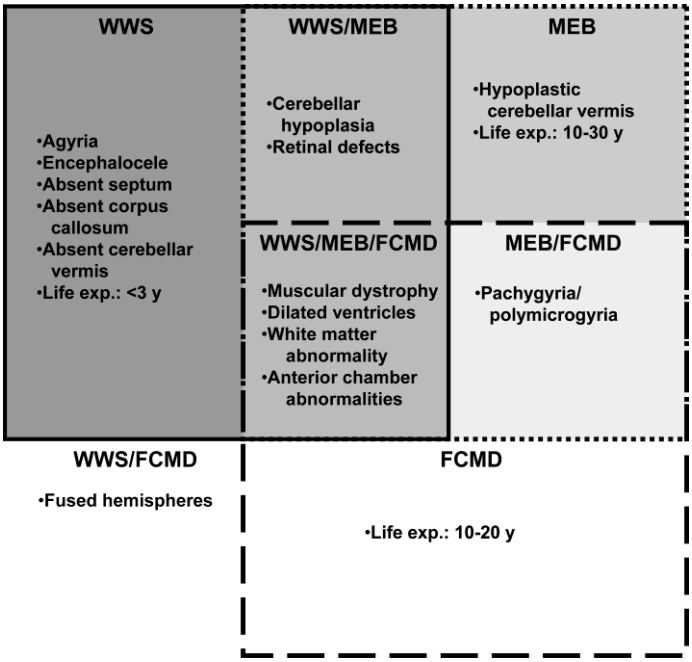

Figure 1. Phenotypes of Warburg syndrome (WWS), muscle-eye-brain disease (MEB) and Fukuyama congenital muscular dystrophy (FCMD). Please note that the severity of the shared characteristics may vary among the three syndromes, usually from severe in WWS to moderate and less severe in MEB and FCMD patients.

In addition to the brain phenotype, patients can have a number of congenital ocular abnormalities, such as cataract, microphthalmia, buphthalmus, persistent hyperplastic primary vitreous and Peters anomaly. WWS patients have little motor activity due to the severe congenital muscular dystrophy (Dobyns et al., 1989; Warburg, 1976; Warburg, 1978). Serum creatine kinase levels are usually well above 1000 U/l. Differentiation between WWS, MEB, and FCMD can be made by examination of the brain architecture using imaging techniques like MRI and CT-scanning or by post-mortem examination (**Figure 1**). The structural eye defects in FCMD patients are generally mild if present at all, which distinguishes these patients from WWS and MEB patients (Cormand et al., 2001; Yoshioka and Kuroki, 1994). On the basis of familial co-occurrence, it was initially suggested that WWS and FCMD are allelic disorders (Toda et al., 1995), but subsequent genetic analyses contradicted that hypothesis (Cormand et al., 2001). It was demonstrated that the three types of cobblestone lissencephaly have a different genetic causation, because a number of WWS families did not map to the known loci for FCMD at 9q31 and MEB at 1p34-p33 (Cormand et al., 1999; Toda et al., 1993). Ironically, now that we know more about the causative genes for cobblestone lissencephaly, it becomes evident that allelism between these three syndromes does occur, albeit rarely.

WWS genetics

It is typical for rare autosomal recessive disorders that most cases originate from populations with a high consanguinity rate. One clear conclusion from attempts at homozygosity mapping for WWS is that the disease is genetically heterogeneous (Beltrán-Valero de Bernabé et al., 2002). This suggests that WWS is the result of malfunctioning of a molecular complex or pathway and not of a single gene disruption. The heterogeneity has precluded the identification of WWS genes by a positional cloning strategy. For this reason, a functional candidate gene approach combined with directed linkage mapping seems to be the only good alternative to identify one or more genes underlying WWS. Clinical similarity with MEB, and the notion that the *POMGnT1* gene encodes a glycosylation enzyme, led to the identification of the first causative gene in WWS (Beltrán-Valero de Bernabé et al., 2002), the *POMT1* gene on chromosome 9q34. Mutations in this gene account for 6/30 WWS patients (20%). Further evidence for genetic heterogeneity in WWS came with the identification of mutations in two other genes. Homozygous nonsense mutations were identified in the *fukutin* gene in two unrelated Turkish families (Beltrán-Valero de Bernabé et al., 2003; Silan et al., 2003). The *fukutin* gene was tested in these families, because it is the causative gene in FCMD. The vast majority of FCMD patients are homozygous for a mild founder mutation in the Japanese population that arose about 3000 years ago (Colombo et al., 2000; Kobayashi et al., 1998). In contrast, the WWS *fukutin* mutations appear to be more severe. *Fukutin* mutations probably contribute very little to the prevalence of WWS. In our series, only three of 30 consanguineous WWS families mapped to this locus and a *fukutin* mutation was identified in only one of them (Beltrán-Valero de Bernabé et al., 2003). The third known WWS gene is *FKRP*, which encodes the fukutin-related protein. Mutations in *FKRP* have also been detected in a number of congenital muscular dystrophies with variable severity (Brockington et al., 2001a; Brockington et al., 2001b; Topaloglu et al., 2003). The clinical spectrum associated with *FKRP* mutations was broadened by the identification of a homozygous missense mutation in a WWS patient of Pakistani origin (Beltrán-Valero de Bernabé et al., 2004). The patient presented typical WWS features and was previously included in a clinical review of MEB and WWS by Cormand et al. (Cormand et al., 2001). Again, mutations in the *FKRP* gene account for only a marginal number of WWS patients as only two of 29 consanguineous patients in our series showed homozygosity at the *FKRP* locus and mutations were found in only one of those two (Beltrán-Valero de Bernabé et al., 2004).

In our experience, causative mutations in the three known WWS genes are found in eight of 40 (20%) patients (Beltrán-Valero de Bernabé et al., 2002; Beltrán-Valero

de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004) (van Reeuwijk, unpublished data). Genome-wide linkage studies in the unexplained families are still in progress, and the number of remaining loci remains unclear. Given the lack of overlapping linkage intervals between individual families, it is entirely possible that three or more genes remain to be identified in WWS (van Reeuwijk, unpublished data).

WWS and related disorders: is there a genotype-phenotype correlation?

The clinical spectrum arising from mutations in genes involved in cobblestone lissencephaly is much wider than previously envisaged. There are several factors that may account for this: (i) the function or step in *O*-linked glycosylation that is catalyzed by the corresponding protein; (ii) the presence of complementing genes; (iii) the severity of the mutation. Information about the first two possibilities is provided in the next section; in this paragraph, we elaborate on the third possibility by providing some examples of a genotype-phenotype correlation.

The clearest genotype-phenotype correlations are seen for *fukutin* gene mutations. The Japanese founder mutation is a 3-kb retrotransposon insertion in the 3'UTR, which results in reduced levels of fukutin mRNA, yet with the capacity to produce a normal protein (Kondo-Iida et al., 1999). Patients who are homozygous for this retrotransposon mutation have the typical FCMD phenotype. The phenotype is significantly more severe in compound heterozygotes carrying the insertion in combination with a nonsense or missense mutation in the other allele (Kobayashi et al., 1998; Kondo-Iida et al., 1999; Saito et al., 2000). WWS is at the most severe end of the phenotypic spectrum and is caused by homozygous nonsense mutations in the *fukutin* gene (Beltrán-Valero de Bernabé et al., 2003; Silan et al., 2003).

A much wider phenotypic spectrum is observed for mutations in the *FKRP* gene (**Figure 2**). Mutations in *FKRP* were first described by Brockington and coworkers in patients with a severe form of congenital muscular dystrophy, denoted MDC1C (Brockington et al., 2001a), and in the much milder limb girdle muscular dystrophy type 2I (LGMD2I) (Brockington et al., 2001b). The clinical manifestation of the muscular dystrophy in LGMD2I patients is quite variable, and homozygous carriers of *FKRP* mutations can be asymptomatic at older age (de Paula et al., 2003). Clinical variability is also seen at the severe end of the spectrum. A patient was reported with severe congenital muscular dystrophy and mental retardation, cerebellar cysts and other cerebellar abnormalities (Topaloglu et al., 2003). Subsequently, *FKRP*

mutations were identified in the gradually more severe MEB and WWS phenotypes. A genotype-phenotype correlation is not obvious for the *FKRP* mutations that are found in the various disorders. Most of the *FKRP* mutations create amino acid substitutions and their functional consequences are hard to predict owing to a lack of knowledge about the function of FKRP protein. In some instances, the effects of missense mutations can be inferred from the associated phenotypes. For example, the homozygous mutation in *FKRP* (Tyr307Asn) that was found in an MEB patient was previously found in heterozygosity in patients suffering from LGMD2I. The less severe mutation in the second allele, Leu276Ile, is a common mutation in patients with LGMD2I and often found in homozygosity in them causing a relatively mild form of LGMD2I. In contrast, the patient with a compound heterozygous mutation of Tyr307Asn and Leu276Ile suffered from quite severe LGMD2I and died in his late teens, suggesting that theTyr307Asn mutation is more detrimental for FKRP function than is the Leu276Ile (Beltrán-Valero de Bernabé et al., 2004).

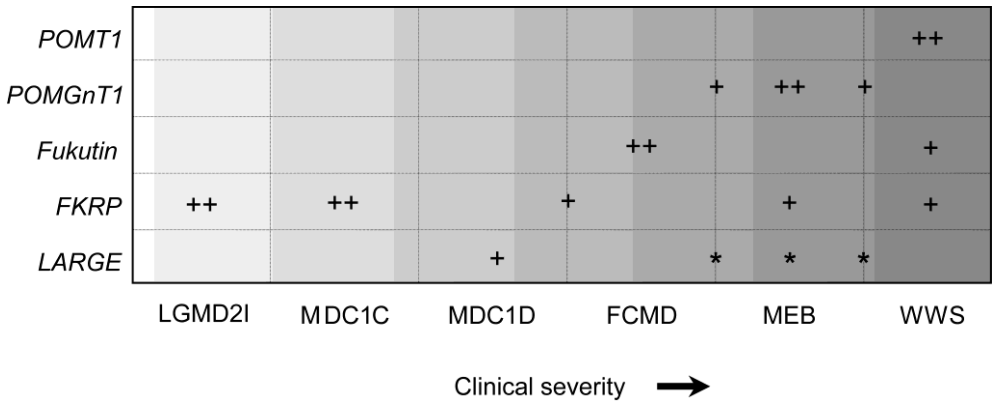

Figure 2. Overview of the genotype–phenotype correlation seen in the cobblestone lissencephalies. The mutations in the genes resulting in the different disorders are depicted by + (rare) and ++ (common). The Large^{myd} mouse phenotype resembles a muscle-eye-brain disease (MEB)/Fukuyama congenital muscular dystrophy (FCMD) phenotype in humans represented by an asterisk (*). *POMGnT1* mutations have been found in patients diagnosed as mild Walker-Warburg syndrome (WWS) and severe FCMD, because their phenotypic characteristics precluded an unequivocal classification into either of these categories.

The final example of a genotype-phenotype correlation is provided by mutations in the *POMGnT1* gene. MEB is most prevalent in the Finnish population due to a founder mutation in the *POMGnT1* gene (de la Chapelle and Wright, 1998). Recently, Toda and coworkers reported a much wider clinical spectrum associated with *POMGnT1* gene mutations, which were identified in patients from all over the world. The mutant POMGnT1 phenotype varied from severe FCMD to MEB to a

WWS-like phenotype, which was less severe than classical WWS. A slight correlation was observed between the clinical severity of the brain phenotype and the location of the mutations in this gene. Mutations near the 5'-terminus of the *POMGnT1*-coding region tend to show more severe brain abnormalities, including hydrocephalus, compared to patients with mutations more downstream who do not have hydrocephalus (**Figure 3**). No differences were observed for the skeletal muscle in which (in both cases) staining of the α-dystroglycan was highly reduced (Taniguchi et al., 2003).

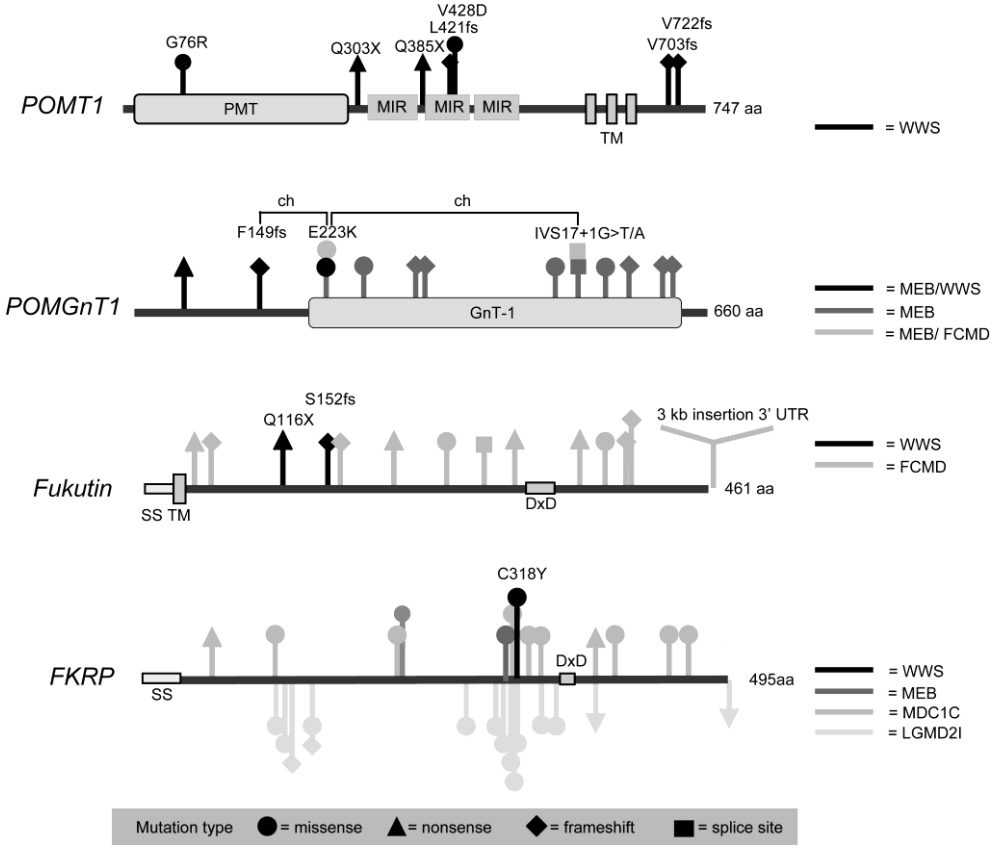

Figure 3. Schematic representation of the proteins involved in cobblestone lissencephalies and the type and position of reported mutations. Compound heterozygosity (ch) is only indicated when associated with an intermediate phenotype. Note that the same missense mutation in *POMGnT1* (E223K) in combination with a truncating mutation at the 5' end (F149fs) gives rise to a more severe phenotype than in combination with a truncating mutation at the 3' end (IVS17+1G>T/A). Protein domains are: PMT, protein mannosyl transferase domain; GnT-1, *N*-acetylglucosaminyltransferase domain; MIR, mannosyltransferase-IP3R-RyR domain; SS, signal sequence; TM, transmembrane domain; DxD, Asp, *X*aa, Asp motif.

As we have seen, mutations in three of the genes involved in cobblestone lissencephaly give rise to a wide phenotypic spectrum, with or without a clear genotype-phenotype correlation (**Figures 2** and **3**). We also hypothesize that mild mutations in *POMT1* might result in the clinically milder disorders such as MEB or FCMD. Indeed, there are still several such patients without a mutation in *POMGnT1*, *fukutin* or *FKRP*. Clinical variability remains to be explored for mutations in the *LARGE* gene. Compound heterozygous mutations in the human *LARGE* gene have been identified in a single patient with a novel type of congenital muscular dystrophy (MDC1D) and severe mental retardation (Longman et al., 2003). Interestingly, the Large^{myd} mouse, which carries an intragenic deletion of exon 4-7 in the *LARGE* gene, has a phenotype that is strikingly similar to the FCMD, MEB and WWS phenotypes in humans (Grewal et al., 2001; Michele et al., 2002; Moore et al., 2002).

Abnormal glycosylation of α-dystroglycan is a key feature of the cobblestone lissencephalies

The *POMT1* gene was selected as a functional candidate for WWS, because of its anticipated role in the post-translational *O*-linked glycosylation of α-dystroglycan (Beltrán-Valero de Bernabé et al., 2002). Currently, there is compelling evidence that shows that the lack of *O*-linked glycosylation of α-dystroglycan is the key defect in WWS and other cobblestone lissencephalies (Endo and Toda, 2003; Grewal and Hewitt, 2003; Martin, 2003; Martin and Freeze, 2003; Martin-Rendon and Blake, 2003; Muntoni et al., 2004). The first line of evidence is genetics. POMT1 is predicted to catalyze the first step in the *O*-linked protein mannosylation, the transfer of a mannosyl residue from dolichyl phosphate mannose to a serine or threonine residue in the target protein (Jurado et al., 1999; Strahl-Bolsinger et al., 1999). POMGnT1 takes care of the second step by adding an *N*-acetylglucosamine residue to a pre-existing protein *O*-linked mannose (Yoshida et al., 2001). The α-dystroglycan protein is the only known substrate for this type of glycosylation in mammals. The second line of evidence is provided by immunohistochemical and functional analyses of α-dystroglycan in material obtained from patients and animal models. There are many reports of strongly reduced or complete lack of glycosylation of α-dystroglycan in material from patients with WWS, MEB, FCMD and related congenital muscular dystrophies (Beltrán-Valero de Bernabé et al., 2002; Brockington et al., 2001a; Brockington et al., 2001b; Brown et al., 2004; Driss et al., 2003; Hayashi et al., 2001; Jimenez-Mallebrera et al., 2003; Longman et al., 2003; Michele et al., 2002; Sabatelli et al., 2003; Topaloglu et al., 2003; Walter et al., 2004). In addition, it was demonstrated that the brain-specific deletion of

α-dystroglycan gives rise to many of the brain malformations that are also seen in WWS, including disorganization of the cortical layers, fusion of cerebral hemispheres, discontinuous pial surface and abnormal migration of post-mitotic neurons (Moore et al., 2002). Finally, also the Large^{myd} mouse has altered glycosylation of α-dystroglycan (Grewal et al., 2001; Michele et al., 2002; Peyrard et al., 1999).

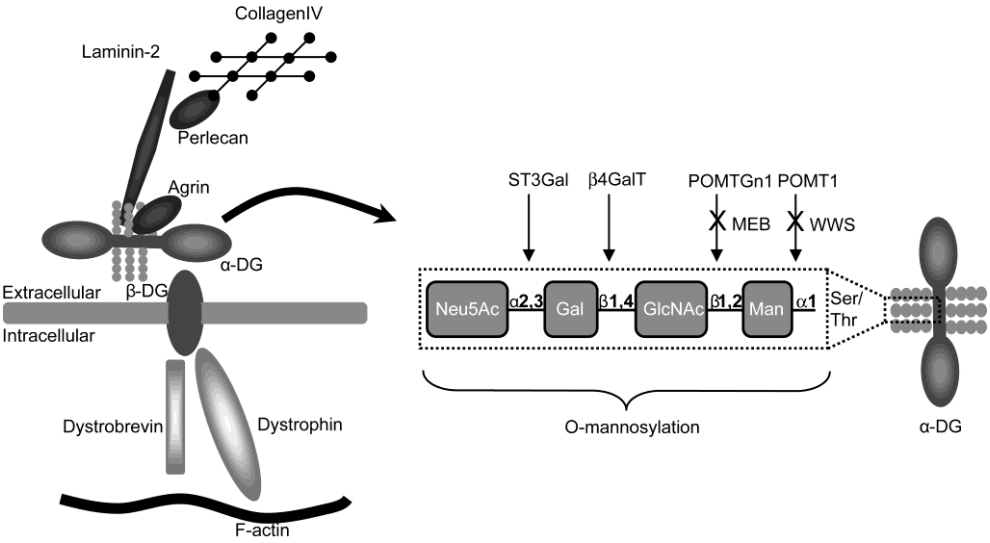

Figure 4. Schematic representation of the crucial role of dystroglycan (DG) in connecting the cytoskeleton of muscle cells with the extracellular matrix. This binding is disrupted by defects in the post-translational modification of α-DG, causing congenital muscular dystrophy. The *O*-mannosylation is represented by small orange spheres. The composition of these carbohydrate side chains and the enzymes that catalyze the addition of these sugar groups are shown in the right panel. Disrupted *O*-mannosylation of α-DG is seen in Walker-Warburg syndrome (WWS) and muscle-eye-brain disease (MEB) patients with mutations in the *POMT1* and *POMGnT1* genes, respectively, which encode the enzymes for the first two steps which cause MEB and WWS when malfunctioning. Similar glycosylation defects are seen in patients with mutations in the *fukutin*, *FKRP* and *LARGE* gene, but the role of the corresponding proteins is not known.

Dystroglycan plays a key role in bridging the cytoskeleton of cells with extracellular matrix (ECM) proteins. Dystroglycan consists of two subunits, α-dystroglycan and β-dystroglycan, which are obtained upon proteolytic cleavage of a precursor protein encoded by a single gene, *DAG1*. Dystroglycan connects the cytoskeleton to the ECM in a number of tissues. In muscle, dystrophin is linked to the actin cytoskeleton and binds the transmembranous β-dystroglycan. The extracellular domain of β-dystroglycan connects the ECM by binding to α-dystroglycan, which in turn binds

to laminin-α2 (Henry and Campbell, 1999) (**Figure 4**). Hypoglycosylation of α-dystroglycan in muscle results in a loss of interaction of this protein with the ECM protein laminin-α2 and thereby disrupts the link between the sarcolemma of the muscle and the ECM (Ervasti and Campbell, 1993; Helbling-Leclerc et al., 1995; Winder, 2001).

The predicted molecular weight of the α-dystroglycan protein is about 72 kDa. However, on immunoblots, the α-dystroglycan protein migrates as a diffuse band of 150-200 kDa, reflecting the extensive post-translational modification of the protein. This increase in molecular weight is largely explained by extensive glycosylation. Both *N*-linked and *O*-linked glycosylation occur. The level and types of glycosylation are likely tissue dependent as judged by different molecular weights. A common glycan structure Neu5Ac(α2-3)Gal(ß1-4)GlcNAc(ß1-2)Man(α1)-Ser/Thr is present at the central mucin region of α-dystroglycan coming from different sources (Chiba et al., 1997; Sasaki et al., 1998; Smalheiser et al., 1998). The Neu5Ac(α2-3)Gal(ß1-4)GlcNAc part of the sugar chain is required for the binding of α-dystroglycan to laminin-α2 (Chiba et al., 1997; Endo, 1999; Yamada et al., 1996). Using antibodies that recognize a carbohydrate epitope, it was demonstrated that cobblestone lissencephaly patients of various types (WWS, MEB and FCMD) have undetectable levels of normally glycosylated α-dystroglycan in skeletal muscle. In contrast, antibodies against the core peptide can detect residual amounts of α-dystroglycan protein. This apparent glycosylation defect of α-dystroglycan is strikingly similar for the various syndromes. Muscular α-dystroglycan shows a reduction of approximately 50 kDa in FCMD and MEB patients as well as in the Large^{myd} mouse. Possibly, failure of glycosylation at any level has the same global effects on the total glycosylation of α-dystroglycan. In agreement with this is the notion that in yeast *O*-mannosylation may be a prerequisite before *N*-linked glycosylation can occur (Ecker et al., 2003). Very recent work demonstrated that overexpression of endogenous *LARGE* in the Large^{myd} mouse diminishes muscular dystrophy by restoring the hypoglycosylation of α-dystroglycan. More strikingly, overexpression of *LARGE* in cells from MEB, FCMD and WWS patients also led to the restoration of functionally glycosylated α-dystroglycan, indicating that LARGE can compensate for the genetically distinct defects in these cells. These data show a possible regulatory role for LARGE in glycosylation of α-dystroglycan (Barresi et al., 2004). It has been established that the molecular interaction between LARGE and the N-terminal domain of α-dystroglycan is crucial for *O*-linked glycosylation of the α-dystroglycan mucin domain (Kanagawa et al., 2004). However, it is as yet unclear

how overexpression of *LARGE* can functionally compensate for the genetic defects in cells from MEB, FCMD and WWS patients (Barresi et al., 2004).

WWS candidate genes

Our genome-wide linkage analyses conducted on 15 consanguineous WWS families point to the existence of at least three further WWS loci (Beltrán-Valero de Bernabé et al., 2002) (van Reeuwijk, unpublished data). Therefore, the functional candidate gene approach in conjunction with homozygosity mapping remains a valid strategy to identify other WWS genes. Our prime targets are other components of the *O*-mannosylation pathway and genes known to result in hypoglycosylation of α-dystroglycan. One excellent candidate gene is the *LARGE* gene. As discussed above, Large^{myd} mice have an altered glycosylation of α-dystroglycan and a phenotype that is strikingly similar to the MEB and WWS in humans (Grewal et al., 2001; Michele et al., 2002; Moore et al., 2002; Peyrard et al., 1999). *POMT2* is another obvious candidate gene, sharing 36% amino acid identity with POMT1. Both genes are expressed in most human tissues, but *POMT1* is expressed mainly in fetal brain, skeletal muscle and testis whereas *POMT2* has its highest expression in testis (Jurado et al., 1999; Willer et al., 2002). In Drosophila melanogaster, orthologues of the human *POMT1* and *POMT2* genes cause the *rt* (rotated abdomen) and *tw* (twisted) phenotypes. These two fly mutants show a similar phenotype with up to 90% rotation of the abdomen resulting from defects in muscle development. In addition, mutant flies have reduced fertility and viability (Martin-Blanco and Garcia-Bellido, 1996; Willer et al., 2003). Another argument for the possible role for POMT2 in WWS is that coexpression of both *POMT1* and *POMT2* is required to obtain POMT enzymatic activity (Manya et al., 2004). This result suggests that a heterocomplex is formed between the two proteins during their synthesis, similar to what has been observed for the homologous PMT1 and PMT2 proteins (Gentzsch et al., 1995; Girrbach and Strahl, 2003). Despite these theoretical considerations, no *POMT2* mutations were identified in 30 unrelated WWS families (Beltrán-Valero de Bernabé et al., 2002). The human genome contains two other genes with some homology to *POMT1*: *SDF2* and *SDF2L1*. Like *POMT1/2*, these two genes encode MIR domains typical of mannosyltransferases, but not the catalytic mannosyltransferase domain. The first two steps in the *O*-mannosyl glycan synthesis of α-dystroglycan are known to be disrupted in cobblestone lissencephalies by mutations in the *POMT1* and *POMGnT1* genes. It is well possible that disruption of other steps in this glycosylation process will give rise to a similar phenotype. The addition of galactosyl (step 3) and the addition of *N*-acetylneuraminic acid (step 4)

are performed by the enzymes β–1,4-galactosyltransferase (β4GalT) and α-2,3-sialyltransferase (ST3Gal), respectively. A frameshift mutation in *B4GALT1* has been implicated in congenital disorder of glycosylation type IId (CDG-IId; MIM 607091). This patient had macrocephaly due to Dandy-Walker malformation, hypotonia, coagulopathy, myopathy with elevated creatine kinase, mild developmental delay, motor retardation and abnormal serum transferrin of which the sialic acid and galactose residues were lost (Peters et al., 2002). The enzyme GNE is known to catalyze a rate-limiting step in sialic acid biosynthesis and defects result in an improper sialylation of glycoproteins (Keppler et al., 1999). Very recently, *GNE* mutations have been linked to hypoglycosylation of α-dystroglycan in patients with hereditary inclusion body myopathy (MIM600737), an autosomal recessive neuromuscular disorder characterized by progressive muscle weakness (Griggs et al., 1995; Huizing et al., 2004). Although further confirmation of this result is required, it indicates that all enzymes involved in *O*-glycosylation of α-dystroglycan are candidates for involvement in neuromuscular disorders. The target proteins for *O*-mannosylation should also be considered for involvement in cobblestone lissencephalies. Currently, the only known *O*-mannosylated protein is α-dystroglycan. We have sequenced the entire coding region of α-dystroglycan in 11 linked WWS patients but found no mutations (van Reeuwijk, unpublished data). Other target proteins for *O*-mannosylation may exist, but could be hard to find because there is no recognizable motif in the proteins that are targets for *O*-mannosylation in yeast. Other proteins from the DGC complex (**Figure 4**), connecting to or present in the ECM in muscle, the central nervous system and the neuromuscular junction are possible WWS candidate genes. An important ligand of α-dystroglycan in the ECM of muscle is laminin-α2 (merosin), mutations of which cause MDC1A (MIM 607855), a common form of CMD and clinically related to FCMD.

Conclusions and perspectives

In the past few years, hypoglycosylation of α-dystroglycan has been found to be the mechanism linking several neuromuscular diseases. This list includes WWS, MEB, FCMD, MDC1C, MDC1D, LGMD2I and some intermediate phenotypes (**Figure 2**). Of these, WWS shows the most severe clinical features including CMD, severe brain and eye involvement. It has been shown that mutations in *POMT1*, *fukutin* and *FKRP* may each give rise to WWS. Knowing that mutations in most of the genes involved in WWS and related disorders show a wide clinical range, mutations in *POMGnT1* and *LARGE* should also be sought in WWS patients. In view of the

genotype-phenotype correlation, we also hypothesize that mild mutations in *POMT1* might cause MEB or even FCMD. It has been shown that POMT1 and POMGnT1 are responsible for the addition of an *O*-linked mannose and a *N*-Acetylglucosamine residue, respectively, to α-dystroglycan (Manya et al., 2004; Yoshida et al., 2001). Elucidation of the exact function of fukutin, FKRP and LARGE in *O*-mannosyl glycan synthesis could lead to further candidate genes for WWS. For this, a better understanding of the post-translational modification of α-dystroglycan will be crucial. Genome-wide homozygosity mapping in consanguineous WWS families may lead to the identification of further genes involved in these disorders. The genetic heterogeneity in WWS and related disorders imposes some difficulties for diagnostic studies and genetic counseling. Presently, mutation analysis for *POMT1* and *POMGnT1* is available in a diagnostic setting (http://www.dnadiagnostieknijmegen.nl), and we emphasize to include the other cobblestone lissencephaly genes in the future. Recently, enzymatic assays have been described to test the activity of POMT1 and POMGnT1 and to diagnose for the founder mutation in FCMD (Kato et al., 2004; Manya et al., 2004; Zhang et al., 2003). These tests in combination with mutation analysis of the known cobblestone lissencephaly genes should allow prenatal diagnosis in selected families.

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Chapter 3

POMT2 mutations cause α-dystroglycan hypoglycosylation and Walker-Warburg syndrome

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Abstract

Walker-Warburg syndrome is an autosomal recessive condition characterised by congenital muscular dystrophy, structural brain defects, and eye malformations. Typical brain abnormalities are hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, cerebellar hypoplasia, and neuronal overmigration, which causes a cobblestone cortex. Ocular abnormalities include cataract, microphthalmia, buphthalmos, and Peters anomaly. Walker-Warburg syndrome patients show defective *O*-glycosylation of α-dystroglycan, which plays a key role in bridging the cytoskeleton of muscle and central nervous system cells with extracellular matrix proteins, important for muscle integrity and neuronal migration. In 20% of the Walker-Warburg syndrome patients, hypoglycosylation results from mutations in either *POMT1*, *FKTN*, or *FKRP*. The other genes for this highly heterogeneous disorder remain to be identified. To look for mutations in *POMT2* as a cause of Walker-Warburg syndrome, as both *POMT1* and *POMT2* are required to achieve protein *O*-mannosyltransferase activity, we combined a candidate gene approach with homozygosity mapping. Homozygosity was found for the *POMT2* locus at 14q24.3 in four of 11 consanguineous Walker-Warburg syndrome families. Homozygous *POMT2* mutations were present in two of these families as well as in one patient from another cohort of six Walker-Warburg syndrome families. Immunohistochemistry in muscle showed severely reduced levels of glycosylated α-dystroglycan, which is consistent with the postulated role for POMT2 in the *O*-mannosylation pathway. A fourth causative gene for Walker-Warburg syndrome was uncovered. These genes account for approximately one-third of the Walker-Warburg syndrome cases. Several more genes are anticipated, which are likely to play a role in glycosylation of α-dystroglycan.

Introduction

Walker-Warburg syndrome (WWS) is a congenital disorder characterised by multiple anomalies of the brain, muscle, and eye. This combination of malformations is also found in muscle-eye-brain disease (MEB) and in Fukuyama congenital muscular dystrophy (FCMD). The most severe anomalies are seen in WWS patients, especially with regard to the brain malformations. Brain malformations typical of WWS are agyria, agenesis of the corpus callosum, cerebellar vermis and septum, and occasional occipital encephalocele (Cormand et al., 2001; Dobyns et al., 1989; van Reeuwijk et al., 2005a). The pathogenesis underlying WWS, MEB, FCMD, and other congenital muscular dystrophies (CMD)

such as congenital muscular dystrophy 1C (MDC1C) and 1D (MDC1D) involves functional disruption of α-dystroglycan by mutations in genes that are involved in *O*-mannosylation of this protein (Dalkilic and Kunkel, 2003; Endo and Toda, 2003; Grewal and Hewitt, 2003; Martin and Freeze, 2003; Martin-Rendon and Blake, 2003; Muntoni et al., 2004; Schachter et al., 2004). The *O*-linked carbohydrate chains of α-dystroglycan are an important component of the dystrophin glycoprotein complex which mediates the interaction between the extracellular matrix and the cytoskeleton of muscle cells and neurons (Kanagawa et al., 2004; Kim et al., 2004; Martin, 2003; Michele et al., 2002; Michele and Campbell, 2003). A common *O*-mannosyl glycan structure found on α-dystroglycan is Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr (Endo, 1999; Willer et al., 2003). The first step in the synthesis of this glycan structure takes place in the endoplasmic reticulum by one or more proteins from a family of well conserved protein *O*-mannosyltransferases (PMTs). These proteins catalyse the transfer of a mannose from dolichyl phosphate activated mannose to serine or threonine residues of secretory proteins. In yeast, a family of seven PMTs (Pmt1p to Pmt7p) are known. Double and triple mutants of the PMTs in yeast indicate that proper *O*-mannosylation is required for its cell wall rigidity and cell integrity (Gentzsch and Tanner, 1996). In Drosophila and human there are only two orthologues: POMT1 and POMT2 (Willer et al., 2003). RNAi knockdown of either *POMT1* or *POMT2* in Drosophila causes a rotation of the abdomen by 30–60˚, demonstrating the requirement of both POMT1 and POMT2 for normal muscle development in the fly (Ichimiya et al., 2004). No embryonic nervous system or eye abnormalities were observed for *POMT1* knockouts (Martin-Blanco and Garcia-Bellido, 1996). In addition, it has been shown that in humans POMT1 and POMT2 are also both required for *O*-mannosyltransferase activity (Ichimiya et al., 2004; Manya et al., 2004). Previously we have shown that mutations in either *POMT1*, *FKTN*, or *FKRP* result in hypoglycosylation of α-dystroglycan, giving rise to the autosomal recessive disorder WWS (Beltrán-Valero de Bernabé et al., 2002; Beltrán-Valero de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004). The phenotypic similarity seen in the fly knockdown of *POMT1* and *POMT2* and the simultaneous requirement for both proteins to obtain *O*-mannosyltransferase in fly as well as human made us reconsider *POMT2* as candidate gene for WWS. In this study we show that *POMT2* mutations also cause WWS.

Methods

Genetic analysis Using standard methods we extracted DNA from peripheral blood lymphocytes. Genome-wide homozygosity mapping was carried out at our linkage facility using the 10 cM spaced microsatellite marker set from Applied Biosystems (ABI Prism linkage mapping set version 2, Applied Biosystems, Foster City, California, USA) and at MRC geneservice in Cambridge with use of the GeneChip Mapping 10K 2.0 arrays from Affymetrix (Santa Clara, California, USA). Additional homozygosity mapping for *POMT2* was undertaken by polymerase chain reaction (PCR) of the flanking microsatellite markers D14S279, D14S983, and D14S59, which were subsequently resolved on 8% polyacrylamide sequencing gels and developed by silver staining.

Mutation analysis All 21 exons of the *POMT2* gene were amplified using specific primers for the 5'- and 3'-flanking intron sequences. Primers and PCR conditions are given in **Table 1**. After purification from agarose gels, the PCR products were used for direct sequencing using the BigDye terminator kit (Perkin Elmer Applied Biosystems, Norwalk, Connecticut, USA), which were analysed on an ABI3700 capillary sequencer. The presence of the identified mutations in the normal population was tested by restriction enzyme analysis in chromosomes from control individuals. For this, the relevant amplicon was digested with TaqI (c.1912C>T), HpyCH4 IV (c.1005+1G>A), and NciI (c.1261delC) (New England BioLabs, Beverly, Massachusetts, USA).

Immunohistochemistry Muscle biopsies from control and WWS patients were obtained after informed consent of patients and approval of the ethics commission. Myoblast cell cultures were established by enzymatic and mechanical treatment of muscle biopsies and by plating in Dulbecco's modified Eagle's medium plus fetal calf serum, penicillin, streptomycin, and amphotericin B (Sigma, Poole, Dorset, UK) (Cenni et al., 2005). Myotubes were obtained by confluent myoblast cultures allowed to differentiate for seven days in cultures medium. Samples for immunohistochemical analysis were fixed in 2% paraformaldehyde in phosphate buffer saline (PBS) and incubated overnight with an anti- α-dystroglycan monoclonal antibody (VIA4-1, Upstate Biotechnology, Lake Placid, New York, USA) diluted 1/50, washed with PBS and then with an antimouse IgG TRITC conjugated antibody (Dako, Glostrup, Denmark). The same sample was then incubated with a polyclonal anti-caveolin 3 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1/50 followed by FITC conjugated anti-goat antibody. The sample was washed in PBS, mounted in Pro-long anti-fade reagent (Molecular Probes, Eugene, Oregon, USA), and examined with a Nikon epifluorescence

microscope at a magnification of x500. Immunohistochemistry on muscle biopsies was carried out as was previously described (Jimenez-Mallebrera et al., 2003). In Brief, frozen 8 mm sections were incubated with primary monoclonal antibodies to β-dystroglycan (NCL-β-dystroglycan, Novocastra Laboratories, Newcastle upon Tyne, UK), α-dystroglycan (IIH6, Upstate Biotechnology), and sheep polyclonal antibody recognizing the core protein of α-dystroglycan (Herrmann et al., 2000). These were than revealed with an appropriate biotinylated secondary antibody (Amersham 1:200; Amersham Life Sciences, Amersham, UK) followed incubation with streptavidin conjugated to Alexa 594 (Molecular Probes) and visualised by epifluorescence microscopy.

Table 1. Primer sequences and conditions for PCR amplification of *POMT2*

Exon	Forward primer	Reverse primer	Product length (bp)	PCR annealing temp (°C)
1	ggagttgcagttccctgagc	taccctcgggccaatcagag	609	58
2	ccatgctttatgaaggccatttg	tggctccagcccttaggaac	224	58
3	gcagctggagggaagttcag	ttagtgtggccccagggttc	235	58
4	tcatcaggtcccctgtcttaaatg	gggcccttgaatgtactgattttc	294	58
5	gtttcctactactggggtgcttgg	acagaaattttggagttgccacag	250	58
6	aagacagggcacagcacagc	ccagaacacagccactctgc	384	58
7	ggctggcccatgtttatcttg	agggtgctggcctttctgag	502	58
8	tccatcacccacctctgtcc	ctttctcccaccgtgccatc	339	58
9	ttagtgagccctgtggcttcc	tgtcatggcgaacagcattg	255	58
10	tggctggggattctgaaattg	tcagcaaagcccatctcagg	304	58
11	ggggtctttcctctttggtctc	tgtggccttgctccattgac	353	58
12	ggtttgggctcatctcttctcc	tccctgtgcagcctcttatcc	403	58
13	gccatttccctttctgacacg	gcagacagcaggtgaacacag	334	58
14	ggaaaagagaaggagcctgtgg	cggagggagtggatgagaag	386	58
15	ctggtgggaatgtggacacc	attcatggctgcccaaaagc	214	58
16	ctgggccccacatttctgtc	cggtctccctgctcttgttc	203	58
17	tcctccccctaatgggtgtg	ggaatgggcagatgagaacg	207	58
18	gcgttctcatctgcccattc	ggtggtaaacgcaaaggatgg	246	58
19	acagcaaggaaggggcagag	tgctctgtctcccaagtccag	263	58
20	cctggctgactccaggtttc	acactgggagggcatgtgag	185	58
21	agctccagcaggaggaatgg	ttccagctgcactcccagag	342	58

Results

Direct linkage mapping of candidate genes using genome-wide homozygosity data We carried out genome-wide homozygosity mapping of 12 WWS patients from 11 unrelated consanguineous families in which linkage to the known WWS loci (*POMT1*, *fukutin*, and *FKRP*) has been excluded. Depending on the amount of DNA available we used the 10 cM spaced microsatellite marker set (ABI Prism linkage mapping set version 2) or the GeneChip Mapping 10K 2.0 Array (Affymetrix). As can be expected from the genetic heterogeneity of WWS, these homozygosity mapping results of mostly singletons failed to point to a single WWS locus and indeed indicated further genetic heterogeneity. Although we failed to detect *POMT2* mutations in an earlier study of 24 WWS families (Beltrán-Valero de Bernabé et al., 2002), we targeted *POMT2* as a candidate gene based on the premise that all three previously identified WWS genes, *POMT1*, *fukutin*, and *FKRP*, are involved in *O*-mannosyl glycan synthesis. A similar function is likely for POMT2, based on the overlapping expression profiles and homologous amino acid composition compared with POMT1. In addition, recent reports indicate that POMT2 is required for the enzymatic activity of POMT1 in human as well as Drosophila (Ichimiya et al., 2004; Manya et al., 2004). Our new mapping data indicated possible linkage to the *POMT2* locus in four of 11 unrelated WWS families.

Mutation analysis of *POMT2* Mutation analysis in all four families led to the identification of mutations in two of these, a nonsense mutation (c.1912C>T, p.R638X) in patient 1 and a splice site mutation (c.1005+1G>A) in patient 2, both found in homozygosity. Both mutations result in the disruption of a restriction site for Taq1 and HpyCH4 IV, in exon 19 and exon 8, respectively. These disruptions were not observed in control individuals digested with Taq1 (170 chromosomes) and HpyCH4 IV (290 chromosomes).

No mutations were detected in the *POMT2* exons in the other two families that showed linkage to the *POMT2* locus. Reasons for this could be that their linkage was a fortuitous finding or that a mutation resides in parts of the *POMT2* gene such as an intron or a regulatory element that have not been analysed. An additional six consanguineous WWS families were then tested for homozygosity at the *POMT2* locus. In two of these WWS families the data were consistent with linkage to *POMT2*. We identified a homozygous 1-bp deletion (c.1261delC) in one these families. This mutation introduces a premature stop codon (p.T433X). The mutation was also homozygously present in an affected sibling and disrupts one of the two restriction sites for NciI in exon 12, which was used to verify the absence of this mutation in 140 control chromosomes from the normal population.

Pedigrees and DNA sequencing results of the three *POMT2* mutated families are shown in **Figure 1**.

Clinical description WWS patients with *POMT2* mutations Patient 1 is a girl born to uncle-niece consanguineous Moroccan parents. A prenatal ultrasound indicated hydrocephalus which was confirmed by magnetic resonance imaging, which also showed type II lissencephaly (**Figure 2**, panels A and B). At birth at 37 weeks gestation, her weight was 3150 grams, and her head circumference was 36 cm. The child was severely hypotonic. Ophthalmological examination showed bilateral Peters anomaly with cataracts, left sided microphthalmia, and right sided buphthalmos. The creatine kinase was greatly raised at 12144 U/l. MRI of the brain documented hydrocephalus, cobblestone lissencephaly, and aplasia of the corpus callosum.

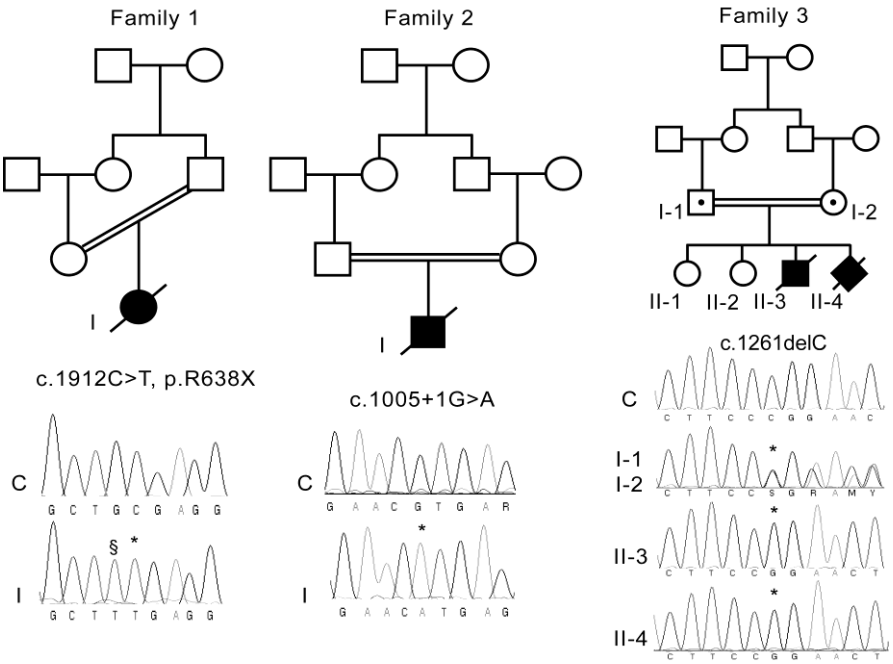

Figure 1. *POMT2* mutations in families with Walker-Warburg syndrome (WWS). For each of the families with *POMT2* mutations the pedigrees and DNA sequencing results are shown. Mutations are indicated with an asterisk (*). Patient 1 is homozygous for a nonsense mutation in exon 19 (c.1912C>T, p.R638X). The mutation in this patient is preceded by a known polymorphism indicated by a § sign. Patient 2 is homozygous for a mutation in intron 8 (c.1005+1G>A) which is predicted (Reese et al., 1997) to result in the disruption of the donor site for intron splicing of intron 8. This mutation also disrupts a restriction site for the HpyCH4 IV enzyme and was not present in 100 chromosomes of controls. Patient 3 is homozygous for a deletion of 1-bp in exon 12 (c.1261delC) which tends to a premature stop codon (p.T433X). The same mutation was found heterozygously in both parents and homozygously in an affected sibling in this family.

Patient 2 is a male child born to first cousin Pakistani parents. Severe hydrocephalus was diagnosed prenatally on ultrasound and the child was delivered by caesarean section. A left-sided cleft lip and palate were diagnosed. Imaging of the brain by MRI revealed aplasia of the posterior vermis, hypoplasia of the pons and cerebellum, severe hydrocephalus, and a cobblestone cortex (**Figure 2**, panels C and D). Ophthalmological investigation documented bilateral cataracts and persistent pupillary membrane. The retina could not be visualised. A muscle biopsy was consistent with a diagnosis of congenital muscular dystrophy. There was increased variability of fibre diameter, increased endomysial fibrosis, and basophilic regeneration. External genitalia were normal. The child died at the age of six months.

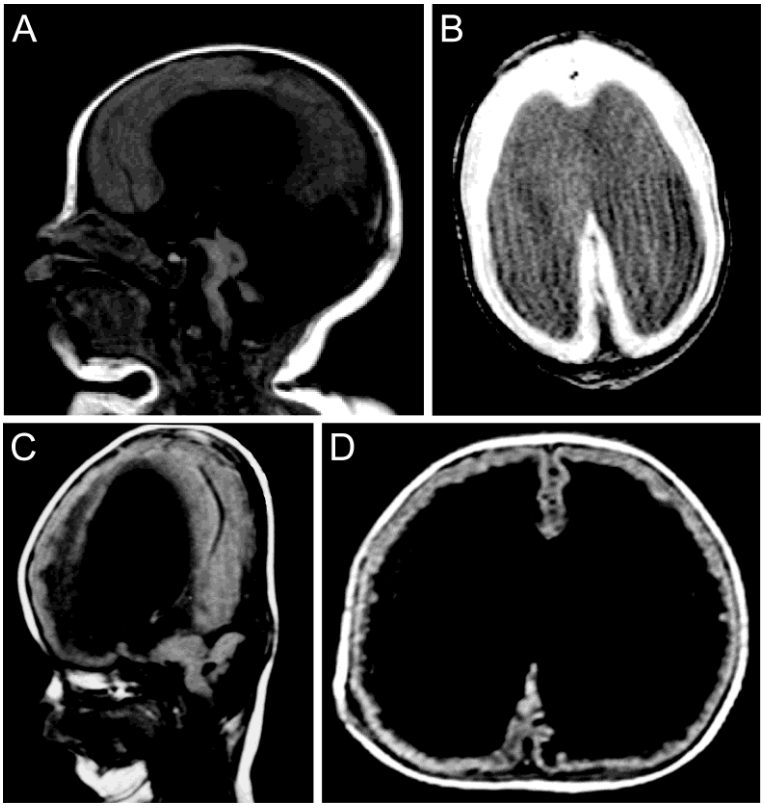

Figure 2. Brain magnetic resonance imaging of patient 1 at two days of age (A and B) and patient 2 at 16 months of age (C and D). Both patients show hydrocephalus, dilatation of the ventricles, agyria, cerebellar hypoplasia, and absence of the corpus callosum.

Patient 3 is a male infant referred at the age of two months and one week because of severe neonatal hypotonia, developmental delay, and poor visual behaviour, as previously reported (Jimenez-Mallebrera et al., 2003). He was the third child of a consanguineous Bengali family. His older siblings were healthy. Congenital hydrocephalus required a shunt at two weeks of age. Serum creatine kinase was markedly raised. Ophthalmological examination showed bilateral lamellar cataracts, and buphthalmos caused by anterior chamber anomalies. He had no head control and no apparent response to visuoacoustical stimuli. Brain MRI at the age of five weeks showed severe hydrocephalus with a thin and smooth cortical mantle. The patient died at eight months of age during a respiratory infection. A further sibling was diagnosed prenatally with severe hydrocephalus at 18 weeks' gestation. This pregnancy was subsequently terminated.

Histopathology of WWS muscle tissue Immunolabelling of the glycoepitope of α-dystroglycan with the VIA4-1 antibody (Ervasti and Campbell, 1991) in cultured myotubes from patient 2 showed markedly reduced staining compared with control myotubes (**Figure 3**, panels A and B). Control labelling with anti-caveolin3, a marker of muscle differentiation, showed that α-dystroglycan co-localises with caveolin3 at the sarcolemma of myotubes derived from a control individual (**Figure 3C**). Similar normal staining was observed in myotubes derived from patient 2 (**Figure 3D**).

Reduced staining of α-dystroglycan was also observed in patient 3 by immunolabelling of the core and glyco-epitope of α-dystroglycan (**Figure 4**, panels D and F). Normal staining was observed for α-dystroglycan in control muscle (**Figure 4**, panels C and E). β-dystroglycan staining was normal in the patient as well as in the control (**Figure 4**, panels A and B). Reduction of immunolabelling of α-dystroglycan and a mild reduction of laminin-α2 in muscle tissue from patient 3 were described previously (Jimenez-Mallebrera et al., 2003). No muscle tissue was available for patient 1.

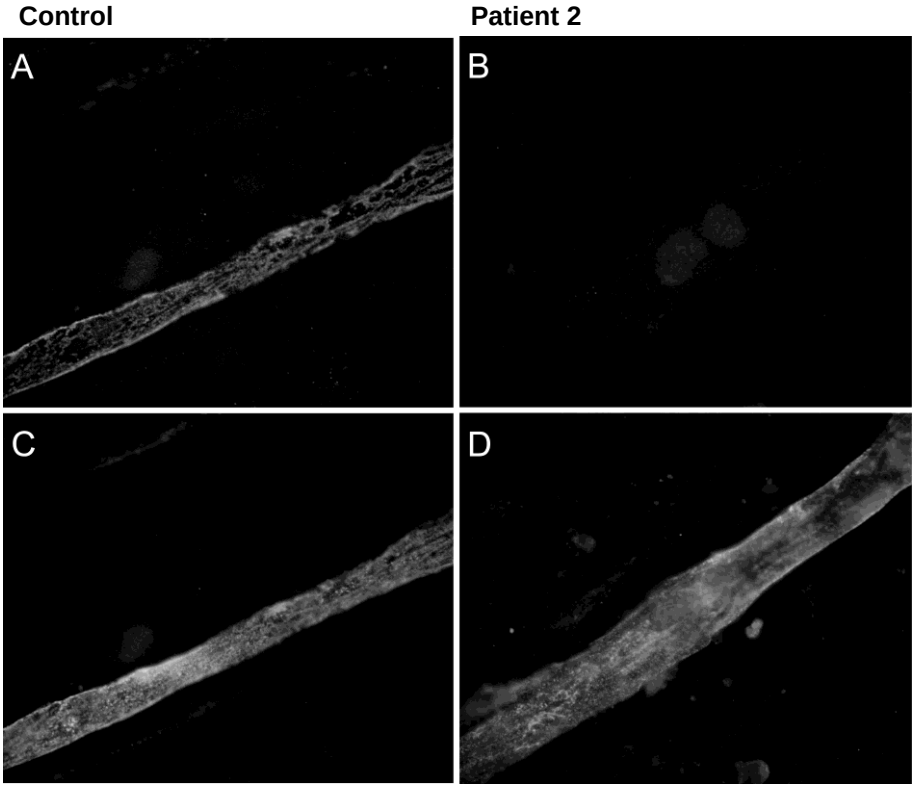

Figure 3. Immunofluorescence analysis of cultured myotubes obtained from a control individual (A and C) and patient 2 (B and D). A double labelling was carried out with anti-α-dystroglycan glycoepitope antibody (VIA4-1) (A and B), and anti-caveolin3, a marker of muscle differentiation (C and D). These were visualised using an anti-mouse TRITC conjugated secondary antibody and an anti-goat FITC conjugated antibody, respectively. In control myotube, α-dystroglycan labelling co-localises with caveolin3 at the sarcolemma. In the WWS patient myotube, caveolin 3 was expressed with a pattern similar to the control myotube while α-dystroglycan staining was severely reduced. Magnification x500.

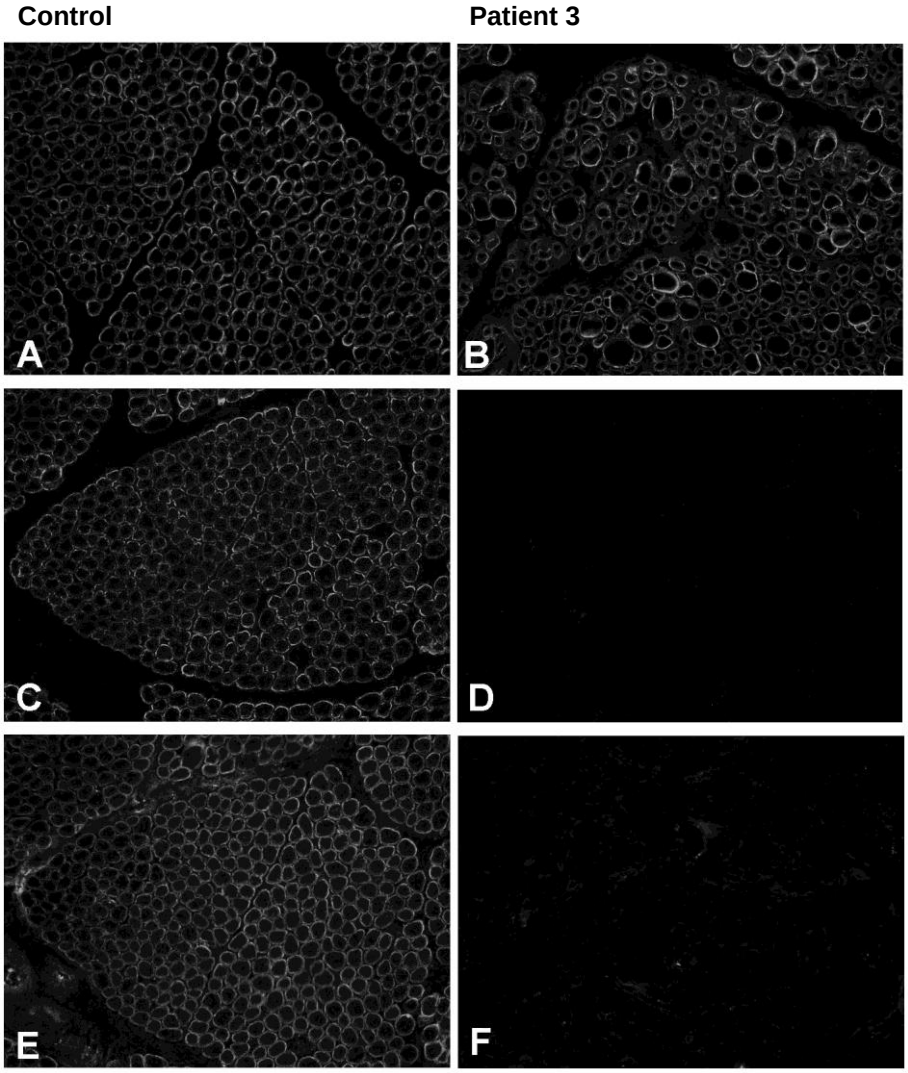

Figure 4. Muscle biopsy of control muscle (A, C, and E) and patient 3 (B, D, and F). Immunostaining with antibodies to β-dystroglycan was normal in both patient and control muscle (A and B). Immunostaining with antibodies that recognise core α-dystroglycan (C and D) and a glycosylated epitope (IIH6) of dystroglycan (E and F) showed normal expression in control but marked reduction of staining in the patient.

Discussion

We have detected three homozygous mutations in *POMT2* in three families with typical WWS. The mutations included a nonsense mutation resulting in a premature stop codon, a splice site mutation, and a 1-bp deletion leading to a premature stop codon. All mutations were homozygous in the patients (**Figure 1**). The phenotype seen in these WWS patients is indistinguishable from that of patients with *POMT1*, *FKTN*, or *FKRP* mutations. One of the sibpair from family 3 who carries a homozygous 1-bp deletion mutation was described earlier, and hypoglycosylation of α-dystroglycan (**Figure 4**) and a possible reduction of laminin-α2 in muscle tissue were documented (Jimenez-Mallebrera et al., 2003). Absence of glycosylated α-dystroglycan was also seen in a muscle biopsy from patient 2 in the present study (**Figure 3**). This is consistent with the postulated role of *O*-glycosylation in normal neuromuscular, brain, and eye development (Michele et al., 2002; Moore et al., 2002). Although previously no enzymatic activity of POMT1 and POMT2 was determined in vertebrates, the involvement of POMT1 in WWS, the high amino acid identity between the two paralogs, and the overlapping expression pattern was reason to hypothesise that mutations in *POMT2* also give rise to WWS (Willer et al., 2002). For this reason we previously undertook mutation analysis of *POMT2* in 24 unrelated patients but no causative mutations were detected (Beltrán-Valero de Bernabé et al., 2002). In this study, 17 additional families where investigated, of which six showed possible linkage to the *POMT2* locus. We found *POMT2* mutations in three of these (**Figure 1**), resulting in a frequency of 7% (3 of 41 families). Thus the incidence of *POMT2* mutations appears to be in the same range as that of *POMT1*. *POMT1* mutations were previously detected at a frequency of 20% and 7% in two large samples of WWS patients (Beltrán-Valero de Bernabé et al., 2002; Currier et al., 2005). This is in accordance with the requirement of both proteins to obtain *O*-mannosyltransferase activity (Ichimiya et al., 2004; Manya et al., 2004). So far, mutations in *POMT1*, *POMT2*, *fukutin*, and *FKRP* together explain almost one-third of the WWS patients in our cohort. The majority of WWS cases remain unexplained and further genetic heterogeneity is likely from our genome-wide homozygosity data.

Acknowledgements

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Chapter 4

Intragenic deletion in the *LARGE* gene causes Walker-Warburg syndrome

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Abstract

Intragenic homozygous deletions in the *Large* gene are associated with a severe neuromuscular phenotype in the myodystrophy (*myd*) mouse. These mutations result in a virtual lack of glycosylation of α-dystroglycan. Compound heterozygous *LARGE* mutations have been reported in a single human patient, manifesting with mild congenital muscular dystrophy and severe mental retardation. These mutations are likely to retain some residual LARGE glycosyltransferase activity as indicated by residual α-dystroglycan glycosylation in patient cells. We hypothesized that more severe *LARGE* mutations are associated with a more severe congenital muscular dystrophy phenotype in humans. Here we report a 63-kb intragenic *LARGE* deletion in a family with Walker-Warburg syndrome, which is characterized by congenital muscular dystrophy, and severe structural brain and eye malformations. This finding demonstrates that *LARGE* gene mutations can give rise to a wide clinical spectrum, similar as for other genes that have a role in the post-translational modification of the α-dystroglycan protein.

Introduction

Abnormal *O*-linked glycosylation of α-dystroglycan is the common pathogenic mechanism in a group of patients with a clinical spectrum ranging from severe congenital muscular dystrophy (CMD), structural brain, and eye abnormalities [Walker-Warburg syndrome (WWS), MIM 236670] to a relative mild form of limb-girdle muscular dystrophy (LGMD2I, MIM 607155) (van Reeuwijk et al., 2005a). In muscle tissue, α-dystroglycan acts as a bridge between the extracellular matrix laminin-α2 and the actin cytoskeleton through the transmembranal β-dystroglycan. The interaction of α-dystroglycan with the extracellular matrix proteins is mediated by its *O*-glycosylated moiety. Mature α-dystroglycan in brain binds to laminin-α2 and neurexin, which is important for neuronal migration in the neocortex and the integrity of the glia limitans (Barresi and Campbell, 2006).

Mutations that result in hypoglycosylation of α-dystroglycan have been identified in known and putative glycosyltransferase genes: *FKTN*, *FKRP*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2* (Freeze, 2006). Four of these genes, *FKTN*, *FKRP*, *POMT1*, and *POMT2*, have been implicated in WWS. These genes explain approximately one-third of the WWS patients in our cohort. Interestingly, different mutations in these genes are known to cause phenotypic variability ranging from WWS to limb-girdle muscular dystrophy. Thus, it appears that mutations in genes that affect the

glycosylation of α-dystroglycan can give rise to a number of related disorders, thereby blurring the boundaries between these clinically defined 'dystroglycanopathies' (Freeze, 2006; Mercuri et al., 2006a; van Reeuwijk et al., 2005a; van Reeuwijk et al., 2006). Here, we have investigated whether allelic variability does also occur for the *LARGE* gene.

The LARGE protein is a putative glycosyltransferase, required for addition of as yet unknown glycans onto the α-dystroglycan protein. Interestingly, overexpression of *LARGE* can by-pass the glycosylation defects in cells from patients with WWS or muscle-eye-brain disease (MEB, MIM 253280) (Barresi et al., 2004). Mutations in *LARGE* have been identified in a patient with relatively mild CMD and severe mental retardation (MDC1D: MIM 608840). This patient is compound heterozygous for p.E509K and p.C667fs. These mutations do not seem to abolish the activity of the LARGE protein completely, as Western blot analysis of skeletal muscle from this patient indicates residual functional glycosylation and laminin-α2 binding activity for α-dystroglycan (Longman et al., 2003). Interestingly, *myd* mice that carry a spontaneous deletion in *Large* ($Large^{myd}$), resemble the more severe clinical Fukuyama congenital muscular dystrophy (FCMD, MIM 253800), and MEB (Grewal et al., 2001; Longman et al., 2003; Michele et al., 2002).

Based on linkage studies we expect that there are multiple other genes that give rise to a WWS phenotype when mutated (van Reeuwijk et al., 2005b) (unpublished data). Here we report a homozygous 63-kb intragenic deletion in *LARGE*, in a patient who had familial classical WWS characteristics. This result establishes *LARGE* as the fifth WWS gene.

Patients and methods

Case reports Patient 1 is a Saudi female and was delivered normally at term. Parents were second-degree cousins. Mother was gravida 5 para 4 with uneventful pregnancy, no polyhydramnios, or reduced fetal movements. Apgar scores were 5 and 8 at 1 and 5 min, respectively. Birth weight 2,880 g (25th centile), length 50 cm (50th centile), head circumference 37 cm (>90th centile). Physical examination (**Figure 1A**) showed no dysmorphic features, severe generalized hypotonia with very little spontaneous movements of the limbs and valgus deformity of the feet. She had poor respiratory effort. Anterior fontanel was wide with separated sutures, deep tendon reflexes were absent, and sensation was intact. Ophthalmic examination revealed dense bilateral congenital cataract in the left eye and mild lens opacity with pigmentary degeneration of the retina and optic atrophy in the right eye. She was

discharged from the Neonatal Intensive Care Unit at the age of 3 months. At the age of 4 months her weight was on the 25th centile, length 10th centile and head circumference above 95th centile. She was developmentally delayed and blind. She died at the age of 6 months.

Laboratory investigations showed remarkably high creatine kinase (CK) of 28,600 U/l (N 24–170) on the third day of life and 1,086 U/l at the age of 1 month. Liver function tests, metabolic screens, and TORCH panel for congenital infections were all normal. Chromosome analysis was normal female karyotype. Brain computed tomography (CT) (**Figure 1B** and **C**) showed marked dilatation of the lateral ventricles with moderate dilatation of the third ventricle, there was a striking decrease in attenuation surrounding the dilated ventricles, the inferior cerebellar vermis was absent, and the cerebellum was hypoplastic. Electroencephalogram showed frequent right tempro-occipital burst of sharp and slow activity. Brain auditory evoked responses using monoaural click stimulation were normal. Muscle biopsy showed features of dystrophy. A diagnosis of WWS was made based on central nervous system, eye, and muscle involvement. No DNA or tissue sample were available for this study.

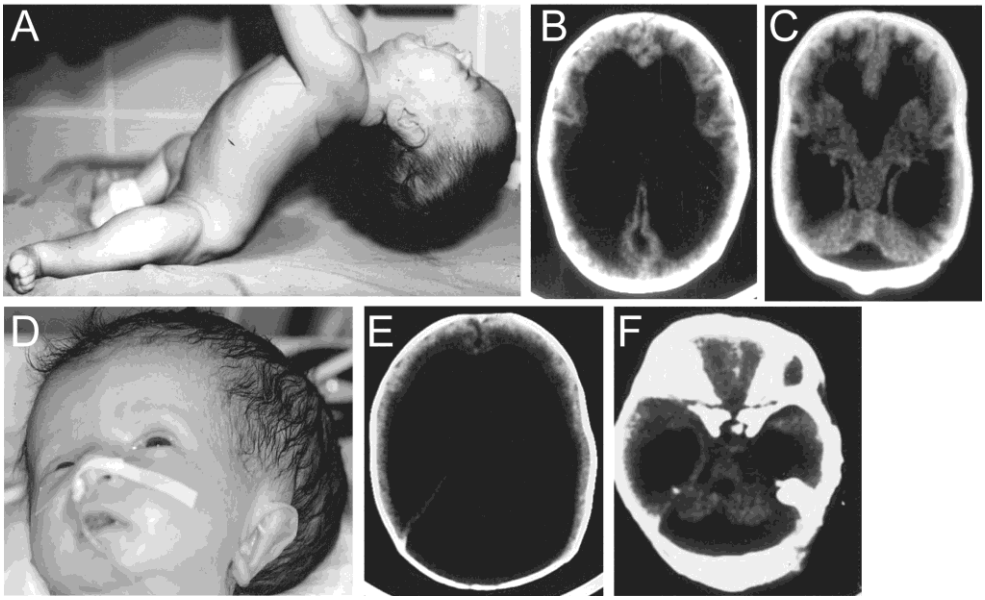

Figure 1. Phenotype and brain CT scans of patient 1 (**A–C**) and patient 2 (**D–F**). Patient 1 showed no dysmorphic features, severe generalized hypotonia with very little spontaneous movements of the limbs, and valgus deformity of the feet (**A**). Brain CT of patient 1 (**B** and **C**), and patient 2 (**E** and **F**) show absence of the inferior cerebellar vermis, a hypoplastic cerebellum, and marked dilatation of the lateral ventricles. Patient 2 also shows severe hydrocephalus with wide fontanel and separated sutures and cysts (**D–F**).

Patient 2 is the younger sibling of patient 1. Antenatal ultrasound showed hydrocephalus and he was delivered by emergency cesarean section. Apgar scores were 6 and 9 at 1 and 5 min, respectively. Physical examination (**Figure 1D**) showed severe hydrocephalus with wide fontanel and separated sutures, and head circumference was 46 cm, above the 97th centile. He had generalized hypotonia, absent deep tendon reflexes but no dysmorphic features. Ophthalmic examination showed bilateral leukocornia, retinal dysplasia, and posterior synechia. Brain CT (**Figure 1E** and **F**) revealed severe hydrocephalus with Dandy-Walker malformation and minimal brain tissue, absent inferior cerebellar vermis and hypoplastic cerebellum. CK was elevated at 18,000 U/l (N 24–195). Lactate dehydrogenase was high at 1,690 U/l (N 230–460). Liver enzymes were mildly elevated, alanine aminotransferase 53 U/l (N 10–50), and aspartate aminotransferase 142 U/l (N 10–45 U/l). Tandem mass spectrometry for metabolic screen was unremarkable; TORCH for congenital infections was negative. Chromosome analysis showed normal male karyotype. Muscle biopsy was done at the age of 11 days and revealed dystrophic features in the form of myofiber necrosis, basophilic fibers and interstitial endomysial, and perimysial fibrosis. There was no specific fiber type atrophy or grouping. A limited number of immunostains were done and included dystrophin and α-sarcoglycan. Both immunostains were normally positive.

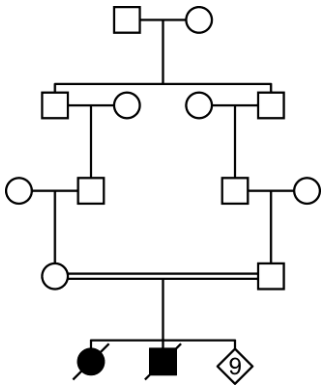

Figure 2. Family pedigree. Females are represented by *circles*, males by *squares*. *Open symbols* represent the unaffected family members, the *solid black symbols* the WWS affected siblings.

A ventriculoperitoneal shunt was inserted. He was weaned of the ventilatory support and died at the age of 2 months. A diagnosis of WWS was made. Nine other siblings were born healthy and DNA was obtained from seven, as well as the parents. A pedigree of the family is given in **Figure 2**.

Linkage analysis and mutation analysis

DNA was extracted from blood lymphocytes using standard procedures. Linkage to the *LARGE* locus was assessed by genotyping two microsatellite markers flanking the gene (D22S281 and D22S529) and two intragenic markers (D22S1162 and D22S1172). Primer3 (http://www.frodo.wi.mit.edu) was used to design specific primers for PCR amplification and direct sequencing of the 14 coding exons (including intron–exon boundaries) of *LARGE* (NM_004737.3).

Copy number detection

Copy number detection of the 16 *LARGE* exons was performed by Multiplex Amplifiable Probe Hybridization (MAPH). A series of probes for the 16 *LARGE* exons were generated by PCR and cloned into pZERO (Invitrogen, Carlsbad, CA, USA). MAPH was carried out and probe ratios analyzed essentially as previously described (Armour et al. 2000; Hollox et al. 2002). Multiplex Ligation-dependent Probe Amplification (MLPA) was used to further restrict the deletion breakpoints. MLPA probes were designed within exons 8–11 and intron 8 and 10 of the *LARGE* gene. A protocol for designing these probes and

hybridization, ligation, and amplification of these probes is provided by MRC-Holland (http://www.mlpa.com). Product separation was performed using capillary electrophoresis on an ABI 3730 or 3100 sequencer (Applied Biosystems, Foster City, CA). For quantitative analysis, trace data were retrieved using Genemapper software following the manufacturer's protocol (Applied Biosystems).

Long-range PCR method and sequence analysis of breakpoints

The breakpoint-spanning region was amplified by long-range PCR amplification with specific primers and LA TaqTM (TaKaRa Bio Inc., Shiga, Japan) using PCR conditions recommended by the manufacturer. We then used different combinations of primers to further restrict the breakpoint-spanning region and identified the breakpoint by direct sequencing.

Results

Linkage to the *LARGE* locus and mutation analysis

We hypothesized that severe *LARGE* mutations give rise to WWS. To assess this hypothesis we selected intragenic and *LARGE* flanking genetic markers to test for homozygosity in 30 WWS patients from consanguineous parents. Seven patients from six unrelated families showed homozygosity for at least two intragenic markers (D22S1162 and D22S1172) and one marker (D22S281 or D22S529) in close proximity of *LARGE*.

We performed mutation analysis for all 14 coding exons including intron–exon boundaries by direct sequence analysis. No mutations were found. However, difficulties during the PCR amplification of some of the exons suggested a possible deletion of these exons in one of the families. Two affected siblings in this family manifest typical WWS features as described in the patients and methods section.

Identification of a submicroscopic deletion by copy number detection
Using MAPH we screened all 16 exons for copy number changes. Exon 9 and 10 showed copy number changes in one family, indicative for a deletion of these exons in patient 2 (homozygous) and in the parents (hemizygous). This result was confirmed by MLPA analysis of probes specific to these exons and surrounding intronic sequences, which also further defined the size of the deletion to 61.1-66.6 kb (**Figure 3**).

Analysis of breakpoints
By long-range PCR amplification we obtained a deletion-spanning fragment of ~3.5-kb from patient 2. Sequence analysis of this fragment revealed the 5' and 3' breakpoints, and a deletion size of 63.1-kb (**Figure 4**). This genomic deletion predicts a 239-bp deletion in the transcript resulting in a shift of the open reading frame within the first predicted catalytic domain, coding for 29 alternative codons followed by a premature stop codon, which most likely results in nonsense-mediated decay of the transcript. Unfortunately no cell-line or tissue sample was available to test this hypothesis. Only the affected individual was homozygous for the deletion, the parents and five of the seven analyzed unaffected sibs were heterozygous carriers of the deletion. No significant match between the 5' and 3' junction sequences is present, therefore the *LARGE* deletion is likely the result of non-homologous end-joining as reported also for the *Large* deletion in *myd* mice (Browning et al., 2005).

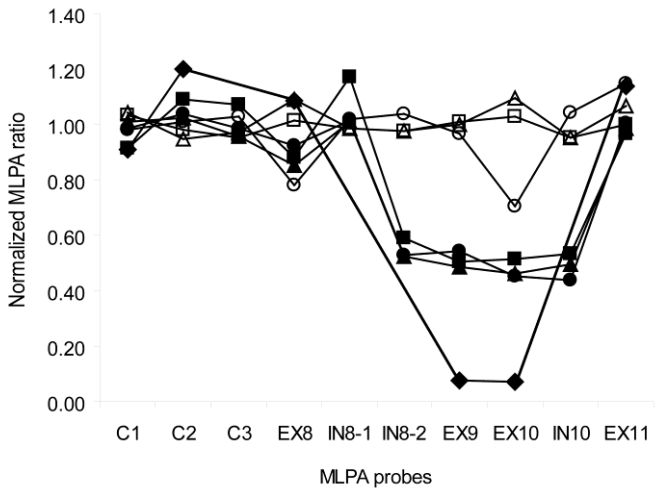

Figure 3. MLPA analysis, showing a deletion of two exonic probes (EX9 and EX10) and two flanking intronic probes (IN8-2 and IN10) in the patient (*diamonds*), and in the unaffected carriers (*black circles, triangles*, and *squares*). No intronic probes were tested for the patient due to limited availability of DNA. *Open circles, triangles*, and *squares* depict control individuals.

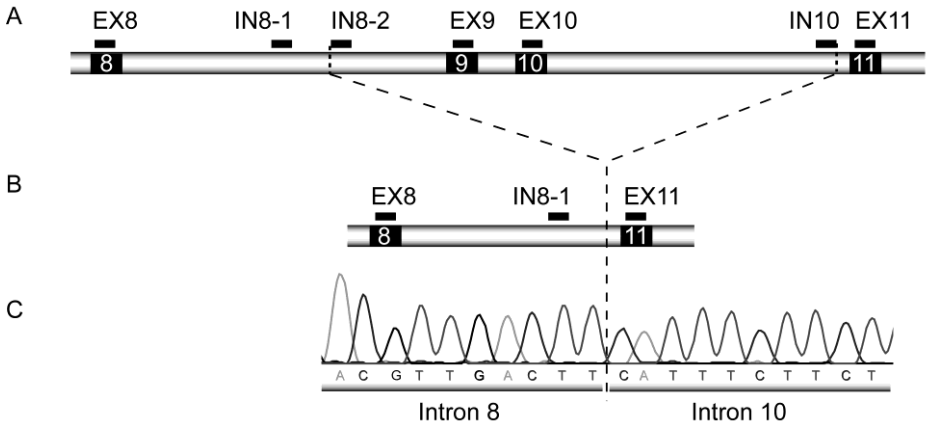

Figure 4. Schematic overview of *LARGE* exons 8–11 of wild-type sequence (A) and patient sequence (B), showing the exons depicted by *black boxes* and the MLPA probes depicted by *black bars*. MLPA analysis revealed deletion of four MLPA probes (IN8-2, EX9, EX10, and IN10). Sequence analysis of the breakpoint region in the patient revealed the exact position of the 63.1-kb deletion (C).

Discussion

We previously reported mutations in *POMT1*, *POMT2*, *FKTN*, and *FKRP* in approximately one-third of the WWS patients in our cohort (Beltrán-Valero de Bernabé et al., 2002; Beltrán-Valero de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004; van Reeuwijk et al., 2005b). Here we report a homozygous 63-kb intragenic deletion in *LARGE* in a patient with WWS. The clinical features of the patients in this family do not diverge from the typical manifestations of other WWS patients. Hence, none of the five WWS genes that are known to date are associated with discriminating clinical features. The deletion described in this report is likely a loss-of-function mutation due to a predicted frameshift of the open reading frame within the first predicted catalytic domain. Mice carrying a similar disruptive defect in the *Large* gene display a severe muscle, eye, and brain phenotype, and have a shortened life span. With regard to the brain defects, these mice have severe neuronal migration defects resulting in a lissencephalic phenotype (Holzfeind et al., 2002; Lee et al., 2005; Michele et al., 2002). The only previously known human *LARGE* mutations (p.E509K and p.C667fs) were identified in a patient with CMD, subtle structural brain abnormalities and severe mental retardation (MDC1D). The less severe clinical phenotype of this patient could be explained by residual activity of the LARGE protein. By an overlay assay, the authors demonstrated that residual α-dystroglycan present in a skeletal muscle biopsy in the

patient retained laminin-α2 binding, whereas this binding is lost in the *myd* mice (Holzfeind et al., 2002; Longman et al., 2003; Michele et al., 2002).

The existence of phenotypic variability for different mutations is also reported for other WWS genes. Mutations in the *POMT1/2* genes were initially identified in WWS (Beltrán-Valero de Bernabé et al., 2002; van Reeuwijk et al., 2005b), but subsequently also in milder conditions including limb-girdle muscular dystrophy subtype 2K (LGMD2K, MIM 609308) (Balci et al., 2005; Mercuri et al., 2006a; van Reeuwijk et al., 2006). Conversely, *FKRP* mutations are a common cause of LGMD, denoted subtype LGMD2I, but rare mutations are also found in severe conditions such as MEB and WWS, two similar disorders with CMD and severe brain and eye malformations (Beltrán-Valero de Bernabé et al., 2004; Brockington et al., 2001b). A common hypomorphic mutation in the *FKTN* gene causes FCMD in the Japanese population (Kobayashi et al., 1998). However, loss-of-function *FKTN* mutations are found in more severe conditions, including WWS (Beltrán-Valero de Bernabé et al., 2003). Finally, different mutations in *POMGnT1* cause phenotypic variability within the MEB disease spectrum (Taniguchi et al., 2003; Yoshida et al., 2001).

LARGE is localized to the Golgi apparatus but the exact function of LARGE is unknown (Brockington et al., 2005; Grewal et al., 2005). It contains two putative catalytic domains, one related to a bacterial glycosyltransferase, and one related to a human glycosyltransferase (Grewal et al., 2001). In addition, LARGE interacts with the N-terminal domain of α-dystroglycan, which is essential for normal glycosylation of this protein (Kanagawa et al., 2004). Another remarkable finding is the therapeutic potential of LARGE, demonstrated by the recovery of dystroglycan processing and functioning in WWS/MEB fibroblasts by overexpression of the *LARGE* gene (Barresi et al., 2004).

Our finding demonstrates the existence of phenotypic variability, especially with regard to the brain, caused by different mutations in *LARGE*. We identified a mutation in this gene in 1 of 30 families, indicating that this gene is causal for only a small percentage of WWS patients. However, two spontaneous mouse mutants for *Large*, both due to intragenic deletions, have been reported in addition to the WWS patient described here (Grewal et al., 2001; Lee et al., 2005). The genomic size of *LARGE* may predispose this gene for genomic deletions. To exclude *LARGE* from genetic involvement in LGMD, or CMD with or without brain involvement it will be important to examine patients for genomic deletions in this gene.

Acknowledgments

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Chapter 5

The expanding phenotype of *POMT1* mutations; from Walker-Warburg syndrome to congenital muscular dystrophy, microcephaly, and mental retardation

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Abstract

The importance of *O*-glycosylation of α-dystroglycan is evident from the identification of *POMT1* mutations in Walker-Warburg syndrome. Approximately one-fifth of the Walker-Warburg syndrome patients show mutations in *POMT1*, which result in complete loss of protein mannosyltransferase activity. Walker-Warburg syndrome patients are characterized by congenital muscular dystrophy with severe brain and eye abnormalities. This suggests a crucial role for α-dystroglycan during development of these organs and tissues. Here we report new *POMT1* mutations and polymorphisms in Walker-Warburg syndrome patients. In addition, we report different compound heterozygous *POMT1* mutations in four unrelated families that result in a less severe phenotype than Walker-Warburg syndrome, characterized by congenital muscular dystrophy with calf hypertrophy, microcephaly, and mental retardation. Compared to Walker-Warburg syndrome patients, these patients have milder structural brain abnormalities, and eye abnormalities were absent, except for myopia in some cases. In these patients we postulate that one or both transcripts for POMT1 confer residual protein *O*-mannosyltransferase activity. Our data suggest the existence of a disease spectrum of congenital muscular dystrophy including brain and eye abnormalities resulting from *POMT1* mutations.

Introduction

Extensive studies on yeast protein mannosyltransferases (Pmt) showed that proper *O*-mannosylation of proteins is required for cell wall rigidity and cell integrity (Gentzsch and Tanner, 1996). Human protein *O*-mannosyltransferase 1 (POMT1; MIM#607423) is closely related to members of the yeast Pmt4 subfamily. POMT1 *O*-mannosyltransferase activity appears to be necessary for the first step in *O*-glycosylation of α-dystroglycan, which is transfer of a mannosyl residue from Dol-P-Man to Ser/Thr residues (Manya et al., 2003; Willer et al., 2003). Homozygous *POMT1* mutations account for Walker-Warburg syndrome (WWS; MIM#236670) in nearly one-fifth of patients, and such patients typically have hypoglycosylation of α-dystroglycan in affected muscle tissue (Beltrán-Valero de Bernabé et al., 2002). WWS patients rarely live beyond the first year of life because of multiorgan involvement. Patients have congenital muscular dystrophy (CMD), eye abnormalities such as cataract, microphthalmia, buphthalmia, and Peters anomaly, and severe structural brain defects. Brain defects include hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, cerebellar

hypoplasia, and neuronal overmigration, which causes a cobblestone cortex (Beltrán-Valero de Bernabé et al., 2002; Cormand et al., 2001; Dobyns et al., 1989; van Reeuwijk et al., 2005a). We have shown that mutations in *POMT2* (MIM#607439), *FKTN* (MIM#607440), and *FKRP* (MIM#606596) also give rise to WWS, in a small percentage of the patients (Beltrán-Valero de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004; van Reeuwijk et al., 2005b). Muscle biopsies from these patients showed similar hypoglycosylation of α-dystroglycan suggesting a common defective pathway. Other CMDs with brain involvement, like muscle-eye-brain disease (MEB; MIM#253280), and Fukuyama congenital muscular dystrophy (FCMD; MIM#253800) reflect other genes involved in this glycosylation pathway and a genotype-phenotype correlation for those genes has been established or is likely to exist (Diesen et al., 2004; Kondo-Iida et al., 1999; Taniguchi et al., 2003; van Reeuwijk et al., 2005a). *POMT1* mutations in WWS patients cause premature stop codons or create amino acid substitutions and deletions in conserved domains. This suggests that these mutations may reflect null alleles (Beltrán-Valero de Bernabé et al., 2002; Kim et al., 2004). In vitro experiments have demonstrated the disruptive effect of these mutations on the *O*-mannosyltransferase activity of POMT1 (Akasaka-Manya et al., 2004). In this study we report the results of *POMT1* mutation analysis in 28 WWS patients. In eight WWS cases we identified seven novel mutations and one previously described mutation. In addition, mutation analysis was performed in patients with a less severe phenotype, consisting of CMD with calf hypertrophy, microcephaly, and severe mental retardation. Causative compound heterozygous mutations were identified in four unrelated families. One family is of Dutch origin. Three families are of Italian origin, including two families that were described earlier (Villanova et al., 2000) and were subsequently labelled "Italian MEB" (Muntoni et al., 2003), although eye abnormalities (severe myopia) were present in only a single patient. In this work, we refer to these four families as congenital muscular dystrophy plus mental retardation (CMD/MR).

Patients, material, and methods

Patients The clinical features as well as brain MRIs for Patients 1 and 2 (first cousins) and Patient 3 from Italian unrelated families are strikingly similar and were described previously (Villanova et al., 2000). In short, these patients have severe mental retardation, microcephaly, hypertrophy of the quadriceps and calf muscle, and structural brain abnormalities (**Figure 1**), but no eye abnormalities. Patients 1 and 2 never developed the ability to walk and are presently 18 and 17 years old. Patient 3 died at the age of 3.5 years from bronchopneumonia due to swallowing problems and aspiration.

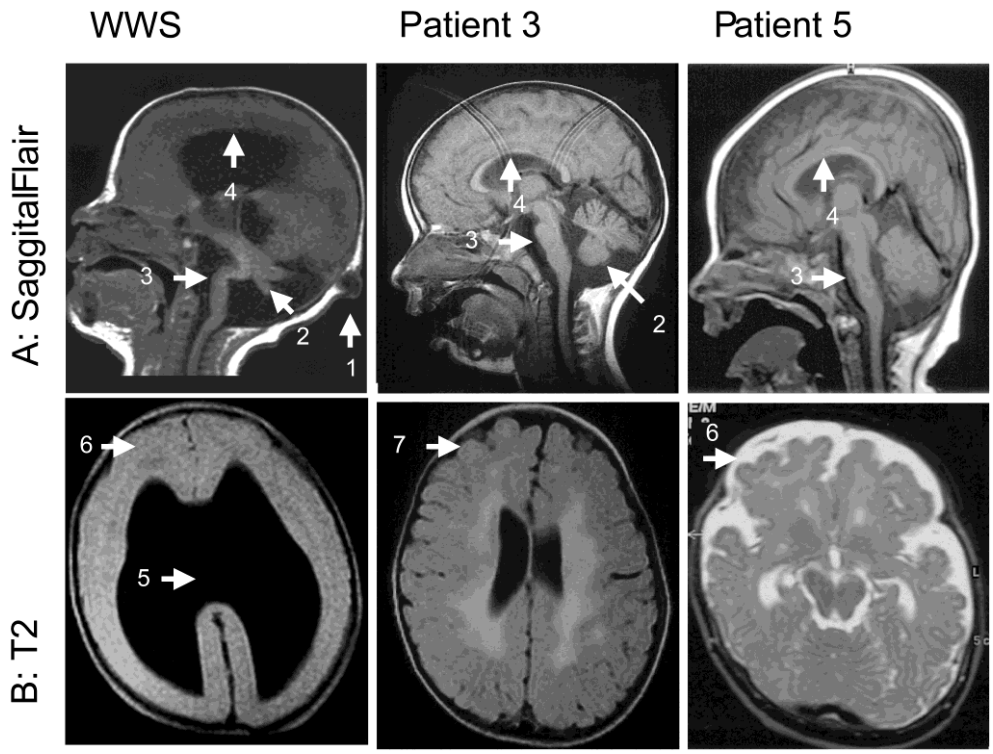

Figure 1. Cerebral MRIs of Patients 3 and 5, compared with a typical WWS patient. Sagittal fluid attenuation inversion recovery MRI (repetition time [TR]=512 msec/echo time [TE]=15 msec) showing hypoplasia of the corpus callosum, brainstem and cerebellum (A). Transverse relaxation time (T2) MRI (TR=3,475 msec/TE=150 msec) shows frontal atrophy and abnormal thickening of the frontal cortex (cortical dysplasia) in the typical WWS patient and in Patient 5, but normal cortex in Patient 3 (B). Arrows indicate the following abnormalities: occipital encephalocele (1); cerebellar hypoplasia (2); hypoplasia of the brainstem (3); hypoplasia of the corpus callosum (4); severe (supratentorial) hydrocephalus (5); abnormal thickening of the frontal cortex (cortical dysplasia) (6); no cortical abnormalities (7).

Patient 4 is a 16-year-old girl of Italian descent born from non-consanguineous parents. She presented at birth, following a normal pregnancy and delivery at term, with hypotonia and microcephaly. Her developmental milestones were characterized by gross delay, with acquisition of the sitting posture at 16 months and inability to walk unsupported. Bilateral hip dislocation was noticed at 2 months and severe myopia at 9 months. The patient had severe mental retardation, was unable to speak, and displayed stereotypic hand-regard movements. Her serum creatine kinase (CK) was markedly elevated at 5,000 U/l (normal value is <200 U/l). Moderate hypertrophy of the thigh muscles was present. A brain MRI performed at the age of 2 years showed megacisterna magna and cerebellar hypoplasia and dilatation of the IV ventricle. The white matter signal was normal and no gross supratentorial cortical dysplasia was visible. At the age of 13 years her head

circumference was 46 cm (below -2.5 standard deviations [SD]); her sitting posture was characterized by mild scoliosis, and flexion contractures of her hips and knees, and equinovarus posture of her feet. A muscle biopsy performed at the age of 5 years was dystrophic with intact laminin-α2 staining. There was no further muscle tissue available to study α-dystroglycan expression.

Patient 5 is a Dutch boy from non-consanguineous parents, who recently died at the age of 4 years. At birth he was hypotonic and contractures were absent. At the age of 4 months, severe visual impairment was suspected. Serum CK level was 6,125 U/l. Ultrasound imaging showed atrophy and increased echodensity of muscles. A muscle biopsy was refused. At the age of 6 months, after the cerebral MRI was done (**Figure 1**) a diagnosis of MEB was made. At the age of 4 years he was microcephalic with a head circumference of 45 cm (below -2.5 SD). Binocular visual acuity (Teller Acuity Cards) was 0.25. There was myopia, astigmatism, and bilateral optic nerve hypoplasia. Ocular pressure was normal, cataract was absent. He had a myopathic face with open mouth. Supine position was frog-like with generalized muscle atrophy, paucity of spontaneous movements, and severe hypotonia with absence of head balance. Flexion contractures of both knees and Achilles tendons were present. Sitting without support was not possible. Patients 6 to 13 are typical WWS patients. **Table 1** gives an overview of the clinical features of the CMD/MR patients (Patients 1 to 5) compared to WWS and MEB patients. Informed consent was obtained from all analyzed subjects.

Linkage analysis DNA was extracted from blood lymphocytes using standard procedures. A genome-wide screening was performed in one family (Patients 1 and 2) using a 400–microsatellite marker set (Applied Biosystems; www.appliedbiosystems.com) at the French National Centre of Genotyping (CNG; www.cng.fr). Linkage to the WWS locus was assessed by genotyping subjects for microsatellite markers flanking *POMT1*, D9S1863, D9S179, and the intragenic marker, D9S64, which is located in intron 2 of the *POMT1* gene (Beltrán-Valero de Bernabé et al., 2002). PCR was carried out with 40 ng of DNA. We used the touchdown PCR method, with annealing temperature decreasing from 65 to 55°C in the first 10 cycles, and fixed at 55°C in the final 20 cycles. All PCRs were done using 0.5 U Platinium Taq polymerase (Invitrogen; www.invitrogen.com) for amplification in a final volume of 15 µl. PCR products were amplified using forward primers labelled at their 5' end with 6-Fam or Hex fluorochromes, and migrated on an ABI 377 automated sequencer. Data were analyzed by the Genscan (version 3.1) Genotyper 2.5 software (Applied Biosystems) and haplotypes were constructed.

Mutation analysis Primer sequences, conditions for PCR amplification, and DNA sequencing of 19 coding exons of *POMT1* (NM_007171.1) in all families was

performed as described previously (Beltrán-Valero de Bernabé et al., 2002). In the case of missense mutations, we determined their causative involvement by checking the Mendelian segregation in family members, and by excluding the presence of these mutations in at least 100 control chromosomes.

Table 1. Clinical features of patients 1-5 (CMD/MR), compared to WWS and MEB patients

Feature	WWS*	MEB*	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Brain							
Cortical abnormalities	Y+++	Y++	Y+	Y+	N	N	Y++
Encephalocele	Y	N	N	N	N	N	N
Hydrocephalus	Y+++	Y++	Y+	Y+	N	N	Y+
White matter abnormalities	Y+++	Y+	Y+	Y+	Y+	N	Y+
Fusion of hemispheres	Y++	N	N	N	Y+	N	N
Septum hypoplasia	Y+++	Y++	N	N	N	N	Y+
Corpus callosum hypoplasia	Y+++	Y+	N	N	N	N	Y+
Cerebellar cortex hypoplasia	Y+++	Y++	Y+	Y++	Y	Y	Y+
Cerebellar vermis hypoplasia	Y+++	Y++	Y+	Y+	Y+	Y	Y+
Speech (words)	N	N	Y+	Y+	Y+	N	N
Mental retardation	Y+++	Y+++	Y++	Y++	Y++	Y++	Y++
Microcephaly	N	N	-3 SD	-3 SD	-3 SD	-2.5 SD	-2.5 SD
Eye							
Microphthalmia	Y++	Y	N	N	N	N	N
Myopia	Y	Y	N	N	Y++	Y	Y
Retinal dysplasia	Y++	Y+	N	N	N	N	N
Muscle							
Serum CK (normal value: 200 U/l)	>5x	>2x	20x	40x	>10x	>10x	30x
Calf hypertrophy	N	N	Y	Y	Y	N (thigh)	Y
Macroglossia	N	N	Y	Y	N	ND	ND
Other							
Able to walk	N	N	N	N	N	N	N
Age (years)	<3	<30	18	17	3.5, †	16	4.5, †

*Clinical features of WWS and MEB patients as previously reported (Beltrán-Valero de Bernabé et al., 2002; Cormand et al., 2001; Taniguchi et al., 2003); Y, observed; N, not observed; +, mild; ++, moderate; +++, severe; ND, no data; CK, creatine kinase; †, deceased

Amino acid conservation In order to determine amino acid conservation of POMT1 in multiple species we made a multiple alignment of human POMT1 orthologous protein sequences using MUSCLE (Edgar, 2004). Proteins with the following GenBank accession numbers were included in the alignment: NP_009102.1 (Human); AAS76201.1 (Mouse); NP_445858.1 (Rat); NP_001025856.1 (Chicken); CAF89480.1 (Fish); AAH75534.1 (Frog); XP_318526.2 (Mosquito); NP_524025.2 (Fly); and NP_596807.1, EAK86491.1, XP_460584.1, EAK95207.1, XP_451701.1, AAS50686.1, NP_012677.1, XP_449354.1, XP_503607.1, EAA64589.1, XP_332024.1, AAP05785.1, and EAA67633.1 (Fungi). A

Clustal colour scheme with a 10% conservation threshold was used to indicate conserved residues in the alignment (Clamp et al., 2004).

Results

Linkage analysis To unravel the genetic basis of two Italian families in which three affected individuals were diagnosed with CMD associated with severe mental retardation, structural brain abnormalities, and muscle hypertrophy, we undertook genome-wide linkage mapping on the most informative family with two patients. Although these patients are first cousins, born to non-consanguineous parents, we hypothesized that a founder mutation may be shared by this and other previously reported Italian families in which linkage to *LAMA2* (MIM#156225), *POMGnT1* (MIM#606822), and *FKTN* was excluded (Villanova et al., 2000). After analyzing several potential loci, no common homozygous markers were found for Patients 1 to 3 and additional patients originating from Italy with similar phenotypes. We then analyzed the genome-wide linkage data based on the hypothesis that Patients 1 and 2 may carry the same compound heterozygous mutations. This allowed the identification of *POMT1* as a candidate, since the two affected individuals shared identical haplotypes for intragenic and *POMT1* flanking markers. Patient 3 appeared to have different haplotypes at the *POMT1* locus (**Figure S1**).

Mutation Analysis *POMT1* mutation analysis was performed in two related (Patients 1 and 2) and 39 unrelated sporadic cases. Twenty-eight cases were diagnosed as WWS, seven cases as intermediate MEB/WWS, and seven cases as CMD including calf hypertrophy, microcephaly, and mental retardation. This last group includes the two Italian families described above (**Figure S1**) and four other families in which no linkage analysis was performed. Four out of these six families, including the two Italian families, revealed causative compound heterozygous mutations for *POMT1* (**Figure 2**). In addition, we detected causative *POMT1* mutations in 7 out of 28 WWS cases. None of the missense mutations was detected in more than 100 chromosomes from control subjects. We found no causative mutations in seven cases of an intermediate MEB/WWS phenotype. All causative *POMT1* mutations identified in this study are listed in **Table 2**. A schematic overview of all causative *POMT1* mutations known to date is given in **Figure 2**. In addition to causative *POMT1* mutations we identified three rare exonic polymorphisms and one intronic polymorphism that have not been described before. An overview of all exonic and rare intronic polymorphisms identified in affected individuals is given in **Table 3**.

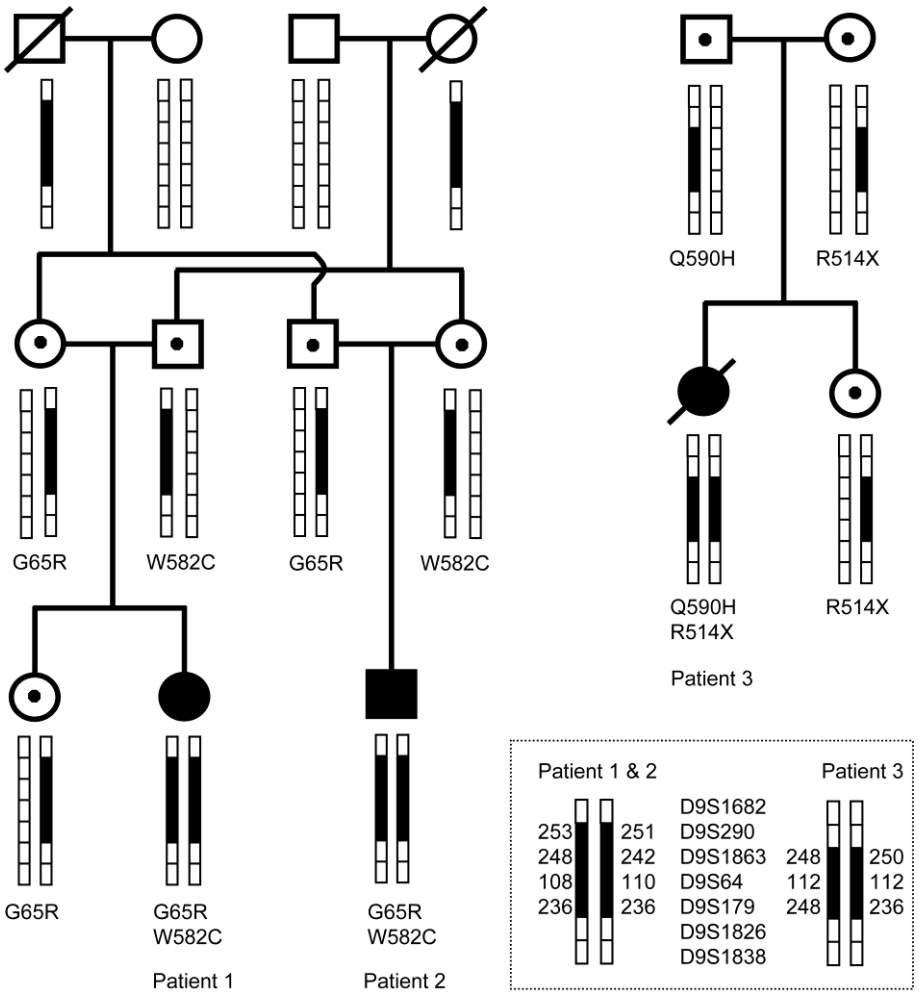

Figure S1. Pedigrees of two Italian families showing haplotypes for chromosome 9q34 polymorphic markers and the segregating *POMT1* mutations. Affected individuals are denoted by blackened symbols and unaffected individuals by unblackened symbols; the deceased family members are indicated by diagonal slashes. The haplotypes segregating with the disease phenotype are shown by black bars with the marker alleles.

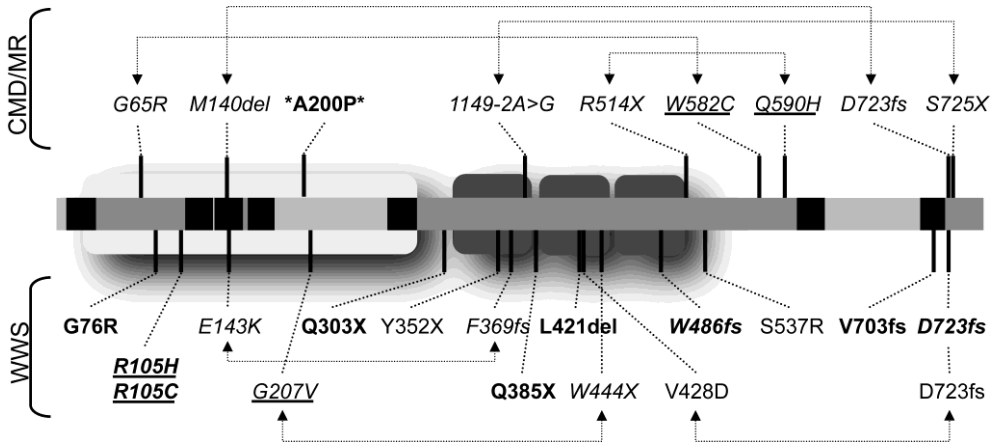

Figure 2. Schematic representation of thePOMT1 protein including all mutations known to date. The highly conserved protein mannosyltransferase (PMT) domain is indicated by a light grey box. Another conserved region is indicated by three dark grey boxes where the mannosyl-IP3R-RyR (MIR) motifs are located. Both conserved elements are thought to be involved in the recognition and/or binding of protein substrates, and/or catalysis. Transmembranous, endoplasmic reticulum (ER), and cytoplasmic parts of the protein are indicated in black, gray, and light gray, respectively. Mutations resulting in WWS are given below the schematic representation of the POMT1 protein while mutations resulting in the milder phenotype are given on top, except for the mutation p.A200P between the asterisks that results in LGMD2K (Balci et al., 2005). Connecting lines between mutations indicate compound heterozygosity and homozygous mutation are in bold. Novel mutations are indicated by italic font. Changes in highly conserved (human-yeast) amino acids are underlined.

Genotype-phenotype correlation for *POMT1* mutations

The expanding phenotype of *POMT1* mutations is suggestive for a genotype-phenotype correlation resulting from mutations that affect transcript or protein in different degrees of severity. We studied whether substituted or deleted amino acids were conserved across multiple species and looked further to see if these amino acids were located in an important domain or 2D structure of the protein. In order to determine the conservation of amino acids we made a multiple alignment of POMT1 orthologous protein sequences from different species (**Figure S2**). The amino acid substitution of highly conserved amino acids (similar residue throughout the alignment) is underlined in **Figure 2**. It is remarkable that most mutations in WWS patients that result in amino acid substitution or deletion are located in the highly conserved PMT and MIR domains, although not all affected amino acids are highly conserved. The only exception is the S537R mutation (Currier et al., 2005).

Table 2. *POMT1* sequence variants and amino acid changes

Patient (Origin)	Diagnosis	Nucleotide variant	Amino acid	Amino acid conservation	Domain	2D structure
1 & 2 (Italy)	CMD/ MR	c.193G>A c.1746G>C	p.G65R p.W582C	++ ++++	PMT No	Coil Coil
3 (Italy)	CMD/ MR	c.1540C>T c.1770G>C	p.R514X p.Q590H	N.A. ++++	3' MIR No	Coil Strand
4 (Italy)	CMD/ MR	c.418_420delATG c.2167dupG*	p.M140del p.D723fs*	+ N.A.	PMT No	Helix Coil
5 (Netherlands)	CMD/ MR	c.1149-2A>G c.2174C>G	(splice site) p.S725X	N.A. N.A.	MIR No	Coil Coil
6 (Netherlands)	WWS	c.427G>A c.1104delC	p.E143K p.F369fs	++ N.A.	PMT MIR	Helix Coil
7 (Pakistan)	WWS	Hom. c.313C>T	p.R105C	++++	PMT	Helix
8 & 9 (Lebanon)	WWS	Hom. c.314G>A	p.R105H	++++	PMT	Helix
10 (India)	WWS	c.620G>T c.1332delG	p.G207V p.W444X	++++ N.A.	PMT MIR	Helix Strand
11 (Qatar)	WWS	Hom. c.1456delT	p.W486fs	N.A.	MIR	Coil
12 (Ireland)	WWS	Hom c.2167dupG*	p.D723fs*	N.A.	No	Coil
13 (Turkey)	WWS	Hom. C.907C>T	p.Q303X	N.A.	No	Helix

*The POMT1 (NM_007171.1) mutations p.Q303X and p.D723fs have previously been identified (p.D723fs as p.G722fs in compound heterozygosity with p.V428D) in WWS patients (Beltrán-Valero de Bernabé et al., 2002). Hom, homozygous, N.A., not applicable, Amino acid conservation of human POMT1 (**Figure S2**) in mammals (+), birds, fish, frogs (++), insects (+++), yeast (++++), PMT, protein mannosyl transferase domain; MIR, Mannosyltransferase; IP3R and RyR domain

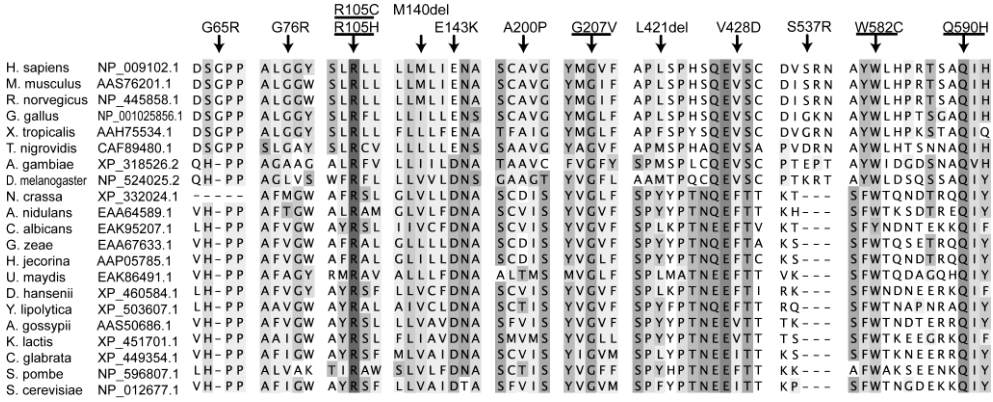

Figure S2. Multiple sequence alignment of POMT1 orthologous protein sequences from different species illustrating the amino acid conservation at different positions in the POMT1 protein that are deleted or substituted as a result of mutations in POMT1 in WWS patients or in CMD/MR patients. Changes of highly conserved residues are underlined.

Table 3. *POMT1* polymorphisms

Nucleotide variant	Amino acid	Amino acid conservation	Domain	2D structure	Reference*
c.718T>G	p.C240G	–	PMT	Helix	rs4997217
c.739A>G	p.M247V	–	PMT	Helix	rs4995933
c.751C>T	p.R251W	–	PMT	Coil	rs3887873
c.752G>A	p.R251Q	–	PMT	Coil	rs2296949
c.855G>C (1%)	**p.L285F**	++	**PMT**	Helix	**This study**
c.942C>T	p.T314T	+++	No	Coil	rs10901065
c.979G>A	p.V327I	+	MIR	Strand	rs4740164
c.1113T>C	p.D371D	++++	MIR	Coil	rs3739494
c.1299C>A	p.D433E	+++	MIR	Coil	rs11243406
c.1565G>A (1%)	**p.R522K**	–	**No**	Coil	**This study**
c.1758G>A	p.R586R	+	No	Coil	Currier et al., 2005
c.1922C>T	p.A641V	+	No	Helix	rs12115566
c.2097C>T (1%)	**p.S699S**	+	**No**	Helix	**This study**
c.1148+16G>A	Intronic	N.A.	N.A.	N.A.	Balci et al., 2005
c.1241+98_99delCT	Intronic	N.A.	N.A.	N.A.	Balci et al., 2005
c.1764+48G>C	Intronic	N.A.	N.A.	N.A.	Balci et al., 2005
c.1764+107A>C	Intronic	N.A.	N.A.	N.A.	Balci et al., 2005
c.1765-6_7CC>AA (3%)	**Intronic**	N.A.	N.A.	N.A.	**This study**
c.2069+13T>C	Intronic	N.A.	N.A.	N.A.	Balci et al., 2005

*Referenced in NCBI SNP Cluster Report. New polymorphisms in *POMT1* (NM_007171.1) are shown in bold with their frequency in between brackets. The changes p.L285F and p.R522K were designated as rare polymorphisms as they were identified only in heterozygosity in consanguineous patients. In addition these are non-conserved residues, which are substituted by a residue that is also present at corresponding positions of orthologous POMT1 protein sequences. N.A., not applicable, amino acid conservation of human POMT1 (**Figure S3**) in mammals (+), birds, fish, frogs (++), insects (+++), yeast (++++), no conservation (–), PMT, protein mannosyl transferase domain; MIR Mannosyltransferase; IP3R and RyR domain

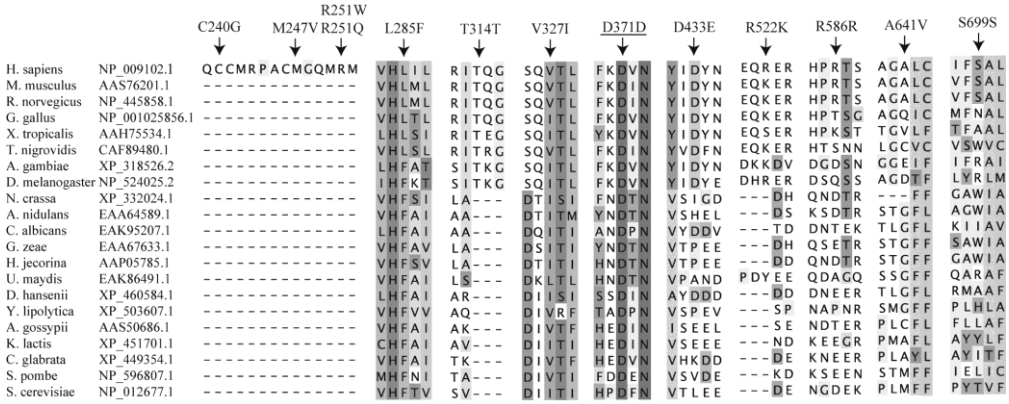

Figure S3. Multiple sequence alignment of POMT1 orthologous protein sequences from different species illustrating the amino acid conservation at different positions in the POMT1 protein. Novel polymorphic changes identified in WWS patients in this study are p.L285F, p.R522K, and p.S699S (silent mutation). Changes of highly conserved residues are underlined.

However, the causation of this S537R change is not proven, because this was the only heterozygous change identified in the *POMT1* gene of a WWS patient. In addition, amino acid S537 is poorly conserved (**Figure S2**). In three patients affected with a milder CMD/MR phenotype (Patients 1, 2, and 4), we found an amino acid substitution (c.193G>A, p.G65R) and deletion (c.418_420delATG, p.M140del) in the PMT domain, but these changes did not involve highly conserved amino acids.

Discussion

Here we describe seven novel *POMT1* mutations, in seven WWS patients that show typical WWS symptoms as described earlier (Cormand et al., 2001; Dobyns et al., 1989). In total we identified *POMT1* mutations in approximately one-fifth of the WWS patients in our cohort, including the patients for which the *POMT1* locus was excluded by haplotype analysis. This is consistent with our previous study, but different from a recent report in which it was shown that the incidence of *POMT1* mutations in WWS patients can be as low as 7% (Currier et al., 2005).

The *POMT1* mutations in WWS patients that are known to date are predicted to be highly disruptive toward the mannosyltransferase activity of the protein, because in nearly all cases they predict truncated proteins or amino acid substitution, or deletion, at critical positions. Indeed, almost complete loss of POMT1 activity has been established for some of the WWS missense mutations (Akasaka-Manya et al., 2004). We therefore postulated that mild mutations in *POMT1* give rise to a milder phenotype than WWS. Haplotype analysis in two unrelated Italian families with three CMD/MR patients (Patients 1, 2, and 3) identified *POMT1* as a candidate gene. Mutation analysis by direct sequencing of *POMT1* in these patients with a less severe phenotype revealed compound heterozygous mutations (c.193G>A, p.G65R; c.1746G>C, p.W582C and c.1540C>T, p.R514X; c.1770G>C, p.Q590H). Direct sequencing of *POMT1* in two more patients, from Italian (Patient 4) and Dutch (Patient 5) origin, with similar clinical features also revealed compound heterozygous mutations (c.418_420delATG, p.M140del; c.2167dupG, p.D723fs and c.1149–2A>G; c.2174C>G, p.S725X, respectively). If our hypothesis is correct then one or both mutations in these CMD/MR patients should be relatively mild, i.e., leading to a mutant POMT1 protein with residual activity. Indeed, the amino acid substitutions and deletions identified in these patients either affect nonconserved residues (p.G65R and p.M140del) or affect amino acids that are located outside the recognizable protein motifs (p.W582C and Q590H). One of the CMD/MR patients (Patient 5) is compound heterozygous for a splice-site mutation (c.1149–2A>G) and

a nonsense mutation (c.2174C>G) in the last exon. Although these appear to be severe mutations, this is not necessarily the case. For example, there are several alternative splice sites close to position c.1149, which would give rise to transcripts in which the reading frame is maintained. Also, the S725X nonsense mutation is the most C-terminal truncation known to date and may yield a protein with residual enzymatic activity. Additional experiments are required to determine the actual effects of the *POMT1* mutations, such as measurement of protein *O*-mannosyltransferase activity in patient cell lines or in transfected cell systems (Akasaka-Manya et al., 2004). Unfortunately, no cell lines are available for the patients described here.

The existence of *POMT1* mutations that result in a relatively mild phenotype is suggestive for a genotype-phenotype correlation for *POMT1* mutations as pointed out previously for other causative genes (i.e., *FKTN*) for WWS and other congenital muscular dystrophies with brain involvement (Diesen et al., 2004; Kondo-Iida et al., 1999; Taniguchi et al., 2003; van Reeuwijk et al., 2005a). Mild *POMT1* mutations may result in even milder disorders of *O*-glycosylation, as recently reported for a homozygous founder missense mutation (c.598G>C, p.A200P) in five Turkish patients with autosomal recessive limb girdle muscular dystrophy (LGMD2K; #MIM 609308) and mild mental retardation (Balci et al., 2005).

Two recent publications demonstrate the simultaneous requirement of POMT1 as well as POMT2 to obtain protein mannosyltransferase activity in human and in Drosophila (Ichimiya et al., 2004; Manya et al., 2004). We recently reported *POMT2* mutations in three WWS patients (van Reeuwijk et al., 2005b). This suggests that mild mutations in *POMT2* may also give rise to a mild phenotype.

Acknowledgements

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Chapter 6

Homozygous *FKRP* start codon mutation causes Walker-Warburg syndrome, the severe end of the clinical spectrum

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Manuscript in preparation for submission

Abstract

Dystroglycanopathies are a heterogeneous group of disorders caused by defects in the glycosylation pathway of α-dystroglycan. The clinical spectrum ranges from severe congenital muscular dystrophy with structural brain and eye involvement to a relatively mild adult onset limb-girdle muscular dystrophy without brain abnormalities and normal intelligence. Mutations have been identified in one of six putative or demonstrated glycosyltransferases. Many different mutations have been identified in *FKRP*, which cover the complete spectrum of dystroglycanopathies. In contrast to the other known genes involved in these disorders, genotype-phenotype correlations are not obvious for *FKRP* mutations. To date no homozygous or compound heterozygous null mutations have been identified in *FKRP*, suggesting that null mutations in *FKRP* could result in embryonic lethality. We report a family with two siblings carrying a homozygous mutation in the start codon of *FKRP* that is likely to abolish FKRP protein function. The clinical phenotype of the patients was consistent with Walker-Warburg syndrome, the most severe disorder in the disease spectrum of dystroglycanopathies.

Introduction

Defective *O*-linked glycosylation of the peripheral membrane protein α-dystroglycan is the common pathophysiological mechanism in a group of disorders, referred to as dystroglycanopathies. The clinical phenotypes range from adult onset limb-girdle muscular dystrophy (LGMD2I, MIM 607155) to early lethal Walker-Warburg syndrome (WWS, MIM 236670). WWS is characterized by a combination of congenital muscular dystrophy (CMD), and structural brain and eye abnormalities (Brockington et al., 2001b; van Reeuwijk et al., 2005a). A specific type of glycosylation, *O*-linked mannose glycosylation is a prerequisite for binding of α-dystroglycan to ligands such as laminin-α2, agrin, and perlecan in muscle, and neurexin in the brain. The only known target for this type of glycosylation is α-dystroglycan, and together with other proteins of the dystrophin-glycoprotein complex it forms a link between extracellular matrix proteins and the actin cytoskeleton (Barresi and Campbell, 2006). Mutations have been reported in six putative or demonstrated glycosyltransferases; *FKRP*, *FKTN*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2*. Initially, each gene was associated with one of the syndromes; *FKRP* mutations giving rise to MDC1C, *FKTN* mutations in FCMD patients, a *LARGE* mutations in a patient with MDC1D, *POMGnT1* mutations in MEB patients, and *POMT1* and *POMT2* mutations in WWS patients (Beltrán-Valero de Bernabé et

al., 2002; Brockington et al., 2001a; Kobayashi et al., 1998; Longman et al., 2003; van Reeuwijk et al., 2005b; Yoshida et al., 2001). However, mutation analysis in patients with milder or more severe syndromes within the dystroglycanopathy spectrum demonstrated clinical variability for different mutations in each of the dystroglycanopathy genes (Godfrey et al., 2007). A wide clinical spectrum is most evident for *FKRP* mutations that were first reported in patients with MDC1C (MIM 606612), subsequently in patients with the milder LGMD2I, and later also in patients with CMD and mild structural brain involvement, in muscle-eye-brain disease (MEB, MIM 253280) and in WWS patients with severe structural brain and eye involvement (Beltrán-Valero de Bernabé et al., 2004; Brockington et al., 2001a; Brockington et al., 2001b; Mercuri et al., 2006b; Topaloglu et al., 2003). Mutations have been identified throughout the *FKRP* coding sequence (http://www.dmd.nl/fkrp_seqvar.html). Most mutations result in amino acid substitutions, and few mutations result in a premature termination codon in compound heterozygosity with an amino acid substitution. A genotype-phenotype correlation is not obvious for most of these mutations of which the functional consequences are hard to predict owing to a lack of knowledge about the function of the FKRP protein (Mercuri et al., 2006b; van Reeuwijk et al., 2005a). There is some evidence that the degree of loss of high molecular weight α-dystroglycan, detected by immunocytochemistry and Western blot analysis in MDC1C and LGMD2 patients, correlates with the clinical severity (Brown et al., 2004; Mercuri et al., 2003; Mercuri et al., 2006b). Also patients with Fukuyama congenital muscular dystrophy (FCMD, MIM 253800), MEB, or WWS caused by mutations in one of the other genes produce a hypoglycosylated form of α-dystroglycan which is less capable of binding to laminin-α2 (Kim et al., 2004; Michele et al., 2002).

Recently a patient with MDC1C was reported to carry a homozygous *FKRP* stop codon mutation, c.1378C>T, Gln460X (Mercuri et al., 2006b). Our group previously reported a homozygous missense mutation (c.953G>A, Cys318Tyr) in a patient with WWS, which is clinically the most severe case reported with *FKRP* mutations. Based on these observations, and the absence of evident loss-of-function mutations in *FKRP*, it was hypothesized that *FKRP* null mutations are not compatible with life (Beltrán-Valero de Bernabé et al., 2004; de Paula et al., 2003; Mercuri et al., 2003; Mercuri et al., 2006b). Here we report a WWS family with two siblings carrying a homozygous start codon mutation in *FKRP*, which most likely results in loss of FKRP protein function.

Patients and methods

Patient 1 The oldest affected sibling, the first child of consanguineous Caucasian parents was born at 37+1 weeks after an elective Caesarean section. At 34 weeks of gestational age severe hydrocephalus was diagnosed by ultrasonography, performed because of diminished moving of the child. The Apgar scores were 6 and 7 after 3 and 5 minutes, respectively. Examination after birth revealed a macrocephalic boy with a birth weight of 2930 gram and a head circumference of 43 cm (>>+2SD). The anterior fontanel was large and bulging with palpable separation of the cranial sutures. The pupils were asymmetric (right 3 mm, left 2 mm) with bilateral absent pupillary reflexes to light. He showed limited spontaneous limb movements mainly of the arms. Tendon reflexes were all hard to elicit. He had no sucking reflex, grasping was present in the hands but absent in both feet. At ophthalmologic examination the right eye appeared to be smaller (cornea diameter OD 8 mm, OS 10 mm). Blood vessels in the iris were dilated. There was bilateral cataract, more pronounced in the right eye. Fundi and intraocular vessels appeared to be normal.

Postnatal brain MRI (**Figure 1**) showed extreme enlargement of the lateral ventricles and the 3rd ventricle with a small malformed 4th ventricle suggestive of aqueduct stenosis. Although interpretation of the cortex was difficult due to severe hydrocephalus, there seemed to be decreased cortical gyration with a cobblestone aspect. Both cerebellar hemispheres, the vermis and the pons were small with abnormal kinking position of the brainstem. The MRI confirmed microphthalmia and revealed persistent hyperplastic primary vitreous of the right eye.

Spine radiography showed no abnormalities. Neonatal hearing screening was abnormal. Serum creatine kinase could not be measured due to a limited blood sample. A muscle biopsy from the quadriceps muscle showed a dystrophic pattern with atrophic and hypertrophic muscle fibers, extensive necrosis of fibers and increase of central nuclei. Immunohistochemistry showed reduced staining of dystrophin 1 with normal staining of sarcoglycans and merosin. Postnatal chromosomal analysis revealed a 46, XY karyotype.

It was decided not to perform neurosurgical intervention and the child died at the age of 6 days due to respiratory failure. Consent was not given for autopsy.

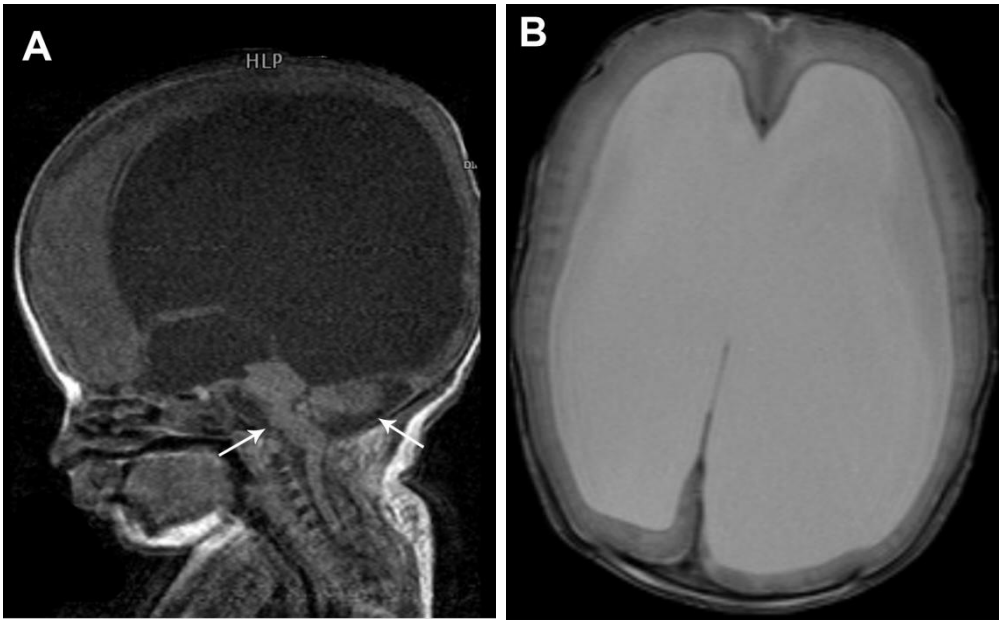

Figure 1. Cerebral MRI of patient 1. **A**: Sagittal T1 showing severe hydrocephalus, hypoplastic brainstem and cerebellar vermis (arrows), and absent corpus callosum. **B**: Axial T2 showing cobblestone lissencephaly and enlarged ventricles.

Patient 2 In the younger affected sibling, severe hydrocephalus was diagnosed at 17+5 weeks of gestation (**Figure 2**). Chromosomal analysis after amniocentesis revealed a normal 46, XX karyotype. At 23+3 weeks of gestation she was born dead after birth induction. Her birth weight was 530 gram. No visible eye abnormalities were seen. Consent was not given for autopsy.

The combination of abnormalities of brain, eye and muscle in patient 1 was indicative for a dystroglycanopathy. Differentiating between MEB and WWS is difficult due to significant clinical overlap. However, MEB patients are predominantly diagnosed in the Finnish population and mostly die after early childhood (Cormand et al., 2001). Most WWS patients die in their first year of life (Dobyns et al., 1989). The clinical phenotype of patient 1, showing extreme prenatal hydrocephalus, severe brain malformation with neuronal migration defect, presence of severe eye abnormalities with microphtalmia and cataract, congenital muscular dystrophy, and the early lethal course is consistent with a diagnosis of WWS (Cormand et al., 2001). The younger female sibling was diagnosed with prenatal hydrocephalus, suggestive for the same diagnosis.

Figure 2. Ultrasound examination of patient 2 at 17+5 weeks of gestation showing severe hydrocephalus.

Genetic analysis DNA was extracted from peripheral blood lymphocytes using standard methods. 10K SNP-array analysis (GeneChip® Mapping 10K 2.0 Array, Affymetrix, USA) was carried out at our linkage facility. Regions ≥10 cM or with ≥20 consecutive homozygous SNPs, and identical genotypes for both affected siblings were defined as regions of homozygosity. Mutation analysis for *FKRP* was performed as described previously (Beltrán-Valero de Bernabé et al., 2004).

Results

The affected siblings were born to consanguineous parents. In order to map the disease causing mutations, we performed genome-wide homozygosity mapping by 10K SNP-array analysis. We identified a homozygous region of 66 contiguous SNP markers on chromosome 19 with identical genotypes in both affected siblings (**Figure 3**). This region of 33 cM in size encompasses the *FKRP* locus. By direct sequencing of the *FKRP* coding DNA we identified a homozygous mutation in the start codon (c.1A>G, Met1Val) in both affected siblings. Both parents were heterozygous carriers of this mutation.

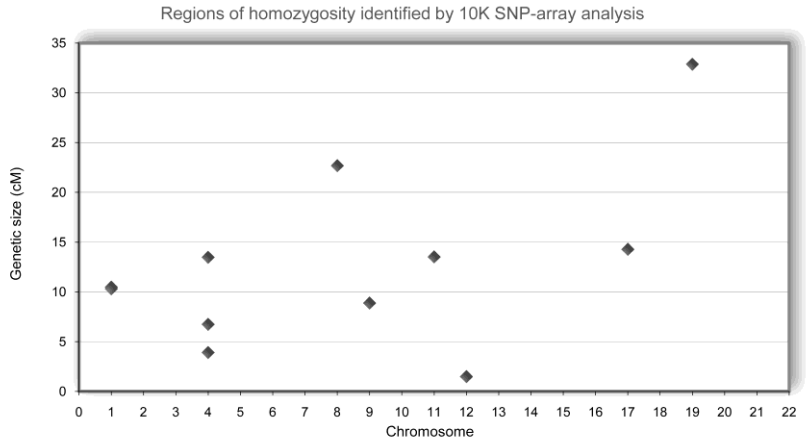

Figure 3. 10K SNP-array analysis identified 10 regions of homozygosity (>=10 cM or with >=20 consecutive homozygous SNPs) with identical genotypes for both affected siblings. The *FKRP* locus resides in the largest region of 33 cM and 66 homozygous SNPs on chromosome 19.

Discussion

Mutations in the *FKRP*, *FKTN*, *LARGE*, *POMT1*, and *POMT2* genes are associated with WWS. The genetic cause for the majority of WWS patients remains unknown. *FKRP* is one of the six known genes (including *POMGnT1*) in which mutations result in defective glycosylation of α-dystroglycan (Brockington et al., 2001a). *FKRP* mutations have been reported to cause a broad spectrum of clinically related muscular dystrophies with severe neurological impairment at the severe end of the spectrum. FKRP-associated disorders include LGMD2I, MDC1C, CMD with mild structural brain involvement, MEB, and WWS (Brockington et al., 2001a; Brockington et al., 2001b; Mercuri et al., 2006b; Topaloglu et al., 2003). Immunocytochemical labeling and Western blot analysis of muscle biopsies from these patients with antibodies that recognize the *O*-glycan moiety of α-dystroglycan (VIA4-1, IIH6) show a variable reduction of α-dystroglycan labeling, which suggests an association between the reduced labeling and the clinical presentation resulting from *FKRP* mutations (Brown et al., 2004; Mercuri et al., 2003; Mercuri et al., 2006b).

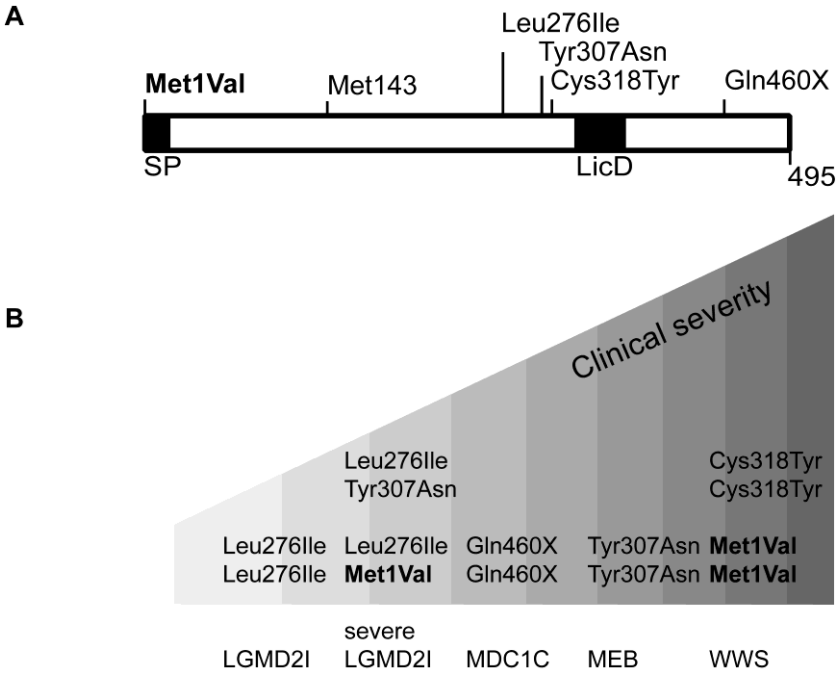

Figure 4. Schematic representation of the FKRP protein and the phenotype associated with different mutations in *FKRP*. **A**: the coding DNA sequence of the *FKRP* gene is contained in one exon. The homozygous Met1Val mutation, identified in a WWS family in this study is shown in bold. Use of an in frame alternative translational start site (Met143) will result in loss of the N-terminal part of the FKRP protein including the signal peptide (SP), which is important for its targeting to the Golgi (Esapa et al., 2002). LicD (Pfam domain PF04991) is found in protein involved in phosphorylcholine metabolism. **B**: different mutations in homozygosity or compound heterozygosity give rise to a range of dystroglycanopathies as described in further detail in the main text.

One of the most common Mendelian mutations in any gene is the *FKRP* missense mutation, c.826C>A (Leu276Ile). The mutation is of European origin, but is found in patients throughout the world (Bushby and Beckmann, 2003). In homozygous state this mutation causes the mildest phenotype observed for *FKRP* mutations, a relative mild form of late-onset LGMD without visible neurological or eye involvement (LGMD2I). Patients who are compound heterozygous for the Leu276Ile mutation show either a slightly more severe LGMD phenotype or even early onset muscular dystrophy (**Figure 4**). Hence, these 'partner' mutations with Leu276Ile are suggested to be more detrimental for FKRP function than the Leu276Ile (Beltrán-Valero de Bernabé et al., 2004; Mercuri et al., 2003). Indeed, the c.919T>A (Tyr307Asn) mutation together with Leu276Ile results in a patient with a severe LGMD2I phenotype who died in his late teens. The severe effect of the Tyr307Asn

mutation was confirmed by its homozygosity in a MEB patient. Likewise, two patients carrying the Leu276Ile mutation in compound heterozygosity with the start codon mutation Met1Val caused a similar severe LGMD2I phenotype as for the Tyr307Asn/Leu276Ile combination (Mercuri et al., 2003; Quijano-Roy et al., 2006). Hence, it is not surprising that a homozygous mutation Met1Val was associated with the severe WWS phenotype in two patients from the WWS family presented here. This start codon mutation may result in the use of an alternative start codon downstream of the coding sequence. The next two potential translational start sites are out of frame. The first alternative in frame translational start site is located 143 codons downstream of the original start codon, and will result in loss of N-terminal residues important for Golgi targeting and retention of the FKRP protein (Esapa et al., 2002). The homozygous Met1Val mutation identified is this study is likely to abolish the FKRP protein function and represents the severe end of the clinical spectrum associated with *FKRP* mutations.

The only other *FKRP* mutation associated with WWS predicts an amino acid substitution Cys318Tyr. The evolutionary conserved cysteine residue appears to be required to form disulphide bridges for protein folding, which are crucial for the function of FKRP. Only one homozygous nonsense mutation, c.1378C>T (Gln460X), has been reported for a patient with MDC1C (Mercuri et al., 2006b), suggesting that this homozygous nonsense mutation is not as severe as the *FKRP* mutations identified in MEB and WWS patients. This may be explained by residual activity of the corresponding mutant FKRP protein, which lacks the last 36 amino acids. Clearly, the Met1Val and Cys318Tyr mutations are more disruptive and likely represent null-alleles. Hence, null mutations in *FKRP* and other genes involved in the *O*-linked glycosylation of α-dystroglycan such as *FKTN*, *POMT1*, *POMT2* and *LARGE*, appear to be associated with the most severe end of the dystroglycanopathy spectrum, WWS. Only for *POMGnT1*, null alleles are associated with a slightly milder phenotype, MEB.

Acknowledgements

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Chapter 7

General discussion and prospects

Introduction

The term “dystroglycanopathies” recently emerged in the literature to denote a growing group of clinically related disorders that result from disruption of the binding of α-dystroglycan to extracellular ligands. No mutations have been found in α-dystroglycan itself and hypoglycosylation of this protein is the only known pathogenic feature common to these disorders. To date, recessive mutations have been identified in six different genes in a variety of clinically distinct dystroglycanopathies. Reported clinical phenotypes range from adult-onset limb-girdle muscular dystrophy (LGMD2I) to early lethal congenital muscular dystrophy with severe structural brain-, and eye abnormalities (Walker-Warburg syndrome [WWS]).

In this chapter I will discuss the implications of the identification of two new WWS genes reported in this thesis, and the clinical spectrum caused by these and other known dystroglycanopathy genes. In addition I will discuss our current diagnostic approach for WWS and related disorders, and future prospects for the identification of new WWS genes.

WWS genetics

Previous studies identified mutations in three WWS genes, *POMT1*, *FKRP*, and *FKTN*, in approximately one-fifth of the WWS patients in our cohort (Beltrán-Valero de Bernabé et al., 2002; Beltrán-Valero de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004). The molecular explanation for a majority of WWS patients remained elusive and genome-wide homozygosity mapping in consanguineous families suggested further genetic heterogeneity (Chapter 2).

In order to identify new WWS loci we performed (genome-wide) homozygosity mapping in additional WWS patients, followed by a candidate-gene-approach. This resulted in the identification of two new WWS genes, *POMT2*, and *LARGE* (Chapters 3 and 4). We considered *POMT2* as a candidate gene for WWS because the involvement of its paralog *POMT1* in WWS, and their overlap in expression pattern. In addition, RNAi knockdown of *POMT1* or *POMT2* in Drosophila shows a striking phenotypic similarity, and both proteins act in concert to obtain *O*-mannosyltransferase activity in Drosophila as well as in human (Ichimiya et al., 2004; Manya et al., 2004). We identified three homozygous *POMT2* mutations in three out of 41 WWS families (7%) (Chapter 3). A previous study by our group failed to identify *POMT2* mutations in a cohort 30 patients, in which six patients (20%) carried a *POMT1* mutation (Beltrán-Valero de Bernabé et al., 2002). A French study

confirmed this lower frequency of *POMT2* mutations (7%), compared to *POMT1* mutations (32%) in a cohort of 47 fetal cases of type 2 lissencephaly (Bouchet et al., 2007). In contrast, another study of 30 WWS patients reveals that the frequency of *POMT1* mutations can also be as low as 7% (Currier et al., 2005). It is not clear why we and others find a difference in mutation frequencies between *POMT1* and *POMT2*. Both proteins are similar in structure and function, and are both together required to obtain *O*-mannosyltransferase activity. Also, common mutations cannot explain the difference.

We considered *LARGE* as a candidate gene for WWS as a defect in the mouse *Large* gene is known to cause the *myd* (myodystrophy) mouse phenotype (Grewal et al., 2001). *Myd* mice have a short life span and show abnormalities very similar to WWS with involvement of muscle, eye, and brain, including lissencephaly. We performed homozygosity mapping of the *LARGE* locus in 30 WWS families, followed by mutation analysis in the six families that linked to this region. In one WWS family with two affected siblings we identified a homozygous intragenic 63-kb deletion (Chapter 4). Like the two deletions found in spontaneous mouse mutants for *Large* (Grewal et al., 2001; Lee et al., 2005) this deletion disrupts the open reading frame of *LARGE*, most likely resulting in a loss of LARGE protein function. An earlier study demonstrated the involvement of *LARGE* compound heterozygous mutations (p.E509K, p.C667fs), in a patient with a milder form of congenital muscular dystrophy, subtle brain abnormalities and mental retardation (MDC1D) (Longman et al., 2003). Several functional studies show the importance of the LARGE protein for *O*-linked glycosylation of α-dystroglycan. LARGE contains two putative catalytic glycosyltransferase domains, and an interaction of LARGE with the N-terminal domain of α-dystroglycan is required for normal glycosylation of this protein. However, the exact function of this Golgi-resident protein remains unknown (Brockington et al., 2005; Grewal et al., 2001; Grewal et al., 2005; Kanagawa et al., 2004). LARGE may be involved in the synthesis of sugar chains other than the *O*-mannosyl sugar chains identified on α-dystroglycan. One of the putative catalytic domains of LARGE is similar to *N*-acetylglucosaminyltransferase and lectin-binding analyses indicate the presence of terminal α-linked GlcNAc (Combs and Ervasti, 2005). This hypothesis is supported by the observations that overexpression of *LARGE* generates highly glycosylated α-dystroglycan even in cells from WWS and MEB patients who have defects in synthesis of *O*-mannosyl sugar chains, which is not observed by overexpression of i.e. *POMGnT1* and *FKRP* (Barresi et al., 2004; Esapa et al., 2002).

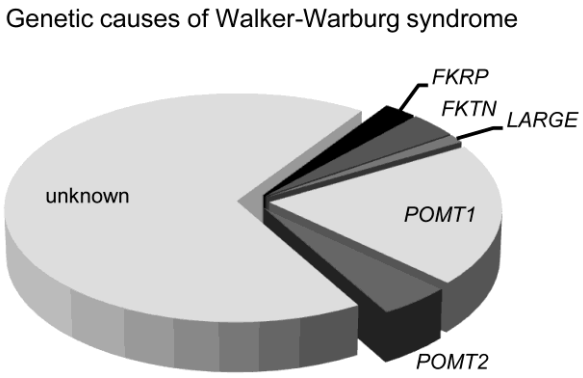


Figure 1. Diagram of genetic causes of WWS. Mutations in *FKRP* (2), *FKTN* (3), *LARGE* (1), *POMT1* (17), and *POMT2* (4) are responsible for WWS in approximately one-third (27/84) of the patients from our research cohort

Mutations in the two new WWS genes, *POMT2*, and *LARGE*, and mutations identified in the three previously identified genes, *POMT1*, *FKRP*, and *FKTN*, together account for approximately one-third of the WWS patients from our cohort (**Figure 1**). Genome-wide homozygosity mapping data from 27 genetically unsolved WWS families provides evidence for extensive genetic heterogeneity (see below).

Clinical spectrum and genotype-phenotype correlations

Mutations in the six genes encoding putative or demonstrated glycosyltransferases, *FKRP*, *FKTN*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2*, are associated with a broad clinical spectrum (**Figure 2**), which is much wider than previously envisaged. Within the group of dystroglycanopathies, phenotypic diversity not only occurs for different mutated genes but also for different mutations within each of the genes. Initially mutations in *POMT1*, and *POMT2* were identified in patients with WWS. Recent work by our group and by others showed that mutations in these genes may also give rise to less severe disorders (Chapter 5) (Balci et al., 2005; Biancheri et al., 2007; D'Amico et al., 2006; Godfrey et al., 2007; Mercuri et al., 2006a; Yanagisawa et al., 2007). Conversely, mutations in *FKRP*, *FKTN*, and *LARGE*, may cause WWS while mutations in these genes were previously identified in clinically less severe disorders (Chapters 4 & 6) (Beltrán-Valero de Bernabé et al., 2003; Beltrán-Valero de Bernabé et al., 2004; Godfrey et al., 2007; Silan et al., 2003).

Mutations identified in *POMGnT1* show the least phenotypic diversity. Most patients manifest a phenotype consistent with muscle-eye-brain disease (MEB). Some variability regarding the brain abnormalities can be noted in some cases, which can be milder or more severe, resembling those seen in FCMD or WWS patients,

respectively (Taniguchi et al., 2003). The only notable exception is a case report of a patient with LGMD and normal intellect, carrying a homozygous missense mutation (p.Asp556Asn) in *POMGnT1* (Clement et al., 2008). A report of a Turkish family provides a strong case for intrafamiliar clinical variability for MEB. Two siblings carrying the same homozygous splice-site mutation in *POMGnT1*, resulting in a deletion of 42 amino acids, show a remarkable difference in severity of the MEB phenotype, suggesting that the phenotype is prone to modification by genetic or environmental factors (Teber et al., 2008). Given the extreme broad clinical spectrum for each of the six known genes, it is impossible to assign the genetic defect to a specific gene, solely on the basis of the clinical characteristics of an individual patient.

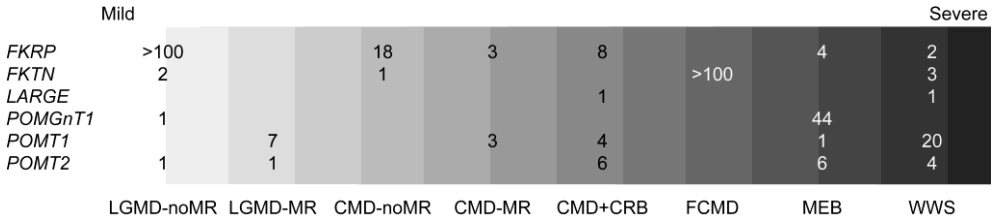


Figure 2. Overview of published mutations (in numbers) in the dystroglycanopathy genes and associated phenotypes ranging from relatively mild limb-girdle muscular dystrophy to severe Walker-Warburg syndrome. Abbreviations: LGMD, limb-girdle muscular dystrophy; MR, mental retardation; CMD, congenital muscular dystrophy; CRB, cerebellar involvement; FCMD, Fukuyama CMD, MEB, muscle-eye-brain disease; WWS, Walker-Warburg syndrome.

Correlations between the predicted effect of a mutation on the protein function, and the resulting clinical features, also referred to as a genotype-phenotype correlation can be observed for some mutations. This is most evident for Walker-Warburg syndrome where nearly all mutations were predicted to completely abolish protein function. These mutations predict either a truncated transcript, or frameshift in the open reading frame, or amino acid substitution or deletion at critical positions of the protein (this Thesis) (Godfrey et al., 2007). Less evident is a genotype-phenotype correlation for *POMGnT1* mutations. Some of the mutations in *POMGnT1* that are predicted to be highly disruptive also show a more severe MEB phenotype, which closely resembles a WWS phenotype (van Reeuwijk et al., unpublished data) (Hehr et al., 2007; Taniguchi et al., 2003; Teber et al., 2008). In contrast, a recent study could not establish a genotype-phenotype correlation for the wider MEB disease spectrum based on POMGnT1 enzyme activity measurements in vitro and in muscle biopsies of patients with novel *POMGnT1* mutations (Hehr et al., 2007). They show that truncating and missense mutations throughout *POMGnT1* uniformly result in complete loss of POMGnT1 enzyme activity, and also report that there is no obvious

correlation between the location of the mutation and the severity of the cerebral malformations, as was reported in a previous study (Taniguchi et al., 2003). Interestingly, the clinically mildest *POMGnT1* mutation known to date, p.Asp556Asn, does show POMGnT1 activity although the kinetics of POMGnT1 activity in the patients' fibroblasts differs from that of control fibroblasts. This residual POMGnT1 activity likely results in the milder phenotype of the patient with LGMD and normal intellect (Clement et al., 2008).

Mutations in *POMGnT1*, *POMT1* and *POMT2* are often associated with the severe end of the spectrum of dystroglycanopathies, such as MEB and WWS, whereas *FKRP* has a higher contribution to the milder end of the clinical spectrum, such as MDC1C and LGMD2I. Multiple factors may play a role in this phenomenon, such as the specific function or step that is disrupted in the catalysis of the *O*-linked mannosyl glycans onto α-dystroglycan or functional redundancy of the mutated protein. Also founder mutations play an important role in this phenomenon. One example is MEB, which is one of the Finnish heritage disorders. Almost all Finnish MEB patients carry the same (homozygous) splice-site mutation (c.1539+1G>A) in *POMGnT1* (Diesen et al., 2004). In Japan, FCMD is the second most common genetic disorder due to a founder mutation in the 3' UTR of *FKTN* (Kobayashi et al., 1998). This mutation has also been observed in Korea, where it may have originated prior to the invasion of Japan from the mainland more than 2000 years ago or more recently from a migrant from Japan to Korea (Colombo et al., 2000; Watanabe et al., 2005). Finally, the Leu276Ile mutation in *FKRP* is a very common mutation in northern Europe with a frequency of 1 in 200 alleles in the Danish and United Kingdom population, and 1 in 600 in the German population (Brockington et al., 2001b; Sveen et al., 2006; Walter et al., 2004). The mutation is of European origin and is found in patients with a mild form of LGMD2I throughout the world. The commonality of this mutation may partly be explained by genetic drift, or an unknown selection pressure to maintain this mutation, or both (Bushby and Beckmann, 2003; Frosk et al., 2005).

WWS diagnostics and genetic counseling

The presence of genetic heterogeneity in WWS and related disorders complicates diagnostic studies and genetic counseling. Mutation analysis is available in a diagnostic setting for all known dystroglycanopathy genes; *FKRP*, *FKTN*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2* (www.dnadiagnostieknijmegen.nl). When parental consanguinity is known or suspected, a prescreen is conducted with genetic markers located in or nearby these genes. Based on homozygosity for these markers, the six

genes may be prioritized prior to laborious mutation analysis. Recently, enzymatic assays have been developed to test enzyme activities of POMGnT1, POMT1, and POMT2 in patient lymphoblast cell lines. If these cell lines are available, these assays provide a noninvasive, and inexpensive method, which can be undertaken prior to mutation analysis (Manya et al., 2008; Vajsar et al., 2006). Hypoglycosylation of α-dystroglycan in muscle biopsies of dystroglycanopathy patients is shown by a reduced immuno-labeling of α-dystroglycan with antibodies to glyco-epitopes of α-dystroglycan (VIA4-1, IIH6) compared to the immuno-labeling with core protein antibodies (GT20ADG). A challenging task remains to identify a serum glycoprotein which is also hypoglycosylated in dystroglycanopathy patients, which would enable a less invasive screening method for dystroglycanopathies. Another possibility for future diagnostics may be the grouping of dystroglycanopathy gene defects based on hypoglycosylation profiles of the molecular mass of α-dystroglycan in patients' lymphoblast or fibroblast cell lines. The major drawback of the diagnostic methods discussed here is their small success rate, since the majority of patients do not carry mutations in any of the six known genes. **Figure 3** shows a scheme for the diagnostic procedure that I propose for (suspected) dystroglycanopathy patients.

Together with the identification of new WWS genes, new methods for mutation analysis need to be developed to facilitate diagnostic testing of multiple genes. Rapid developments in sequencing technologies may facilitate specific sequencing of WWS genes in a more time and cost efficient manner (Albert et al., 2007; Hodges et al., 2007; Okou et al., 2007; Porreca et al., 2007). Second generation sequencing technologies, such as Roche-454 (www.454.com), Illumina-Solexa (www.illumina.com), and ABI-SOLiD (www.appliedbiosystems.com), facilitate sequencing of up to gigabase amounts in a single run. However, these high-throughput technologies still require enrichment of target sequences and extensive bioinformatic analysis, which presently precludes their practical application in a diagnostic setting.

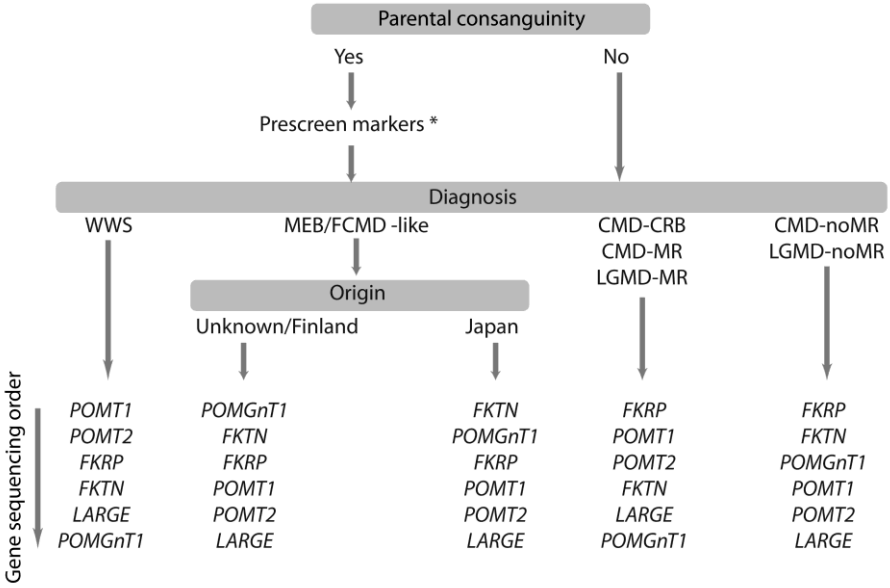


Figure 3. Proposal workflow gene sequencing of dystroglycanopathies based on mutation frequencies for the different clinical categories. Clinical categories are defined as described previously (Godfrey et al., 2007). Abbreviations: WWS, Walker-Warburg syndrome; MEB, muscle-eye-brain disease; LGMD, limb-girdle muscular dystrophy; CMD, congenital muscular dystrophy; CRB, cerebellar involvement; MR, mental retardation. *prescreen with genetic markers in or surrounding the six genes for initial exclusion of genes for mutation analysis based on heterozygosity.

Unravelling the unknown genetic causes of WWS

With the identification of mutations in six genes involved in WWS and related disorders we and others obtained more insight into the etiology of these disorders. The pathomechanism shared by these disorders is disruption of the synthesis of *O*-linked mannose glycans found on α-dystroglycan (**Figure 4**). However, homozygosity mapping, direct mutation analysis or both excluded the involvement of the six known dystroglycanopathy genes in a majority of the WWS patients in our research cohort. Based on our genome-wide homozygosity mapping data from 27 families we predict the existence of several other WWS loci (see below). This implies the involvement of several other crucial components in the *O*-mannosylation pathway of α-dystroglycan. We performed homozygosity mapping, direct mutation analysis or both in our research cohort for obvious candidates from this pathway

such as the gene encoding dystroglycan (*DAG1*), and the genes encoding β–1,4-galactosyltransferases (*B4GALT*1-*B4GALT*7) and α-2,3-sialyltransferases (*ST3GAL1-ST3GALT6*) that catalyse the addition of galactosyl and *N*-acetylneuraminic acid, respectively, to the major *O*-linked oligosaccharide found on α-dystroglycan, Neu5Ac(α2-3)Gal(ß1-4)GlcNAc(ß1-2)Man(α1)-Ser/Thr. Although the results were negative for these genes, we cannot exclude their involvement in dystroglycanopathies. Similar as for *POMT2* it is possible that more patients need to be tested before disease causing mutation are identified. In any case, these genes will not have an important contribution to the prevalence of WWS.

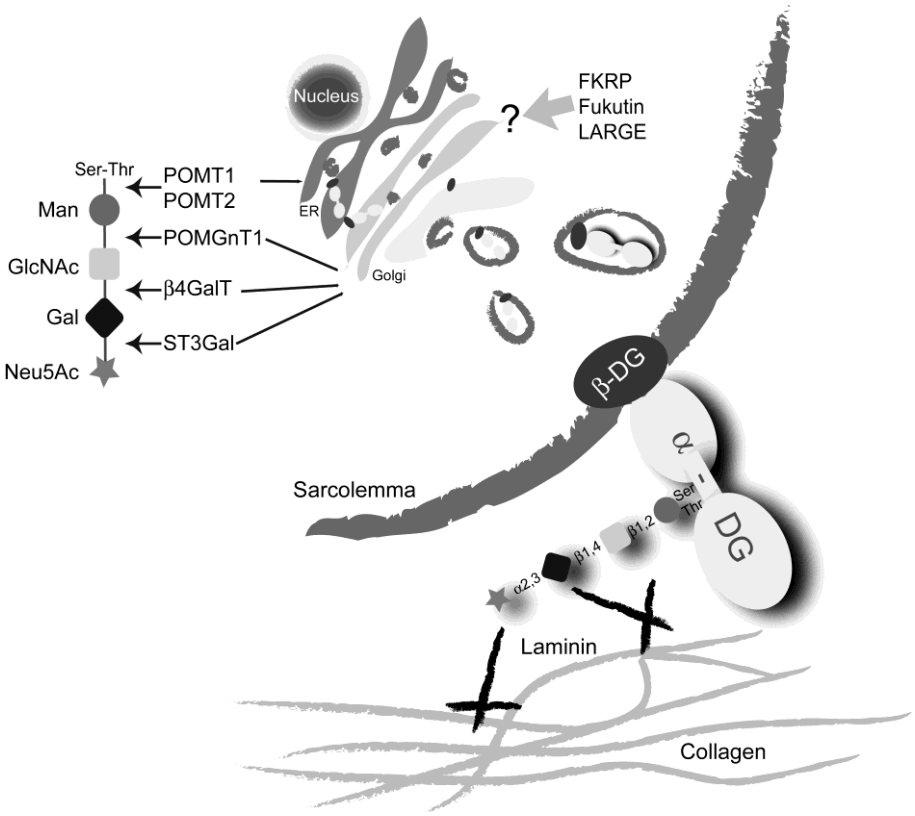


Figure 4. Glycosylation of α-dystroglycan. Localization of glycosyltransferases involved in the *O*-glycosylation pathway of α-dystroglycan within the cellular organelles. Known steps of the glycosylation pathway are represented on the left.

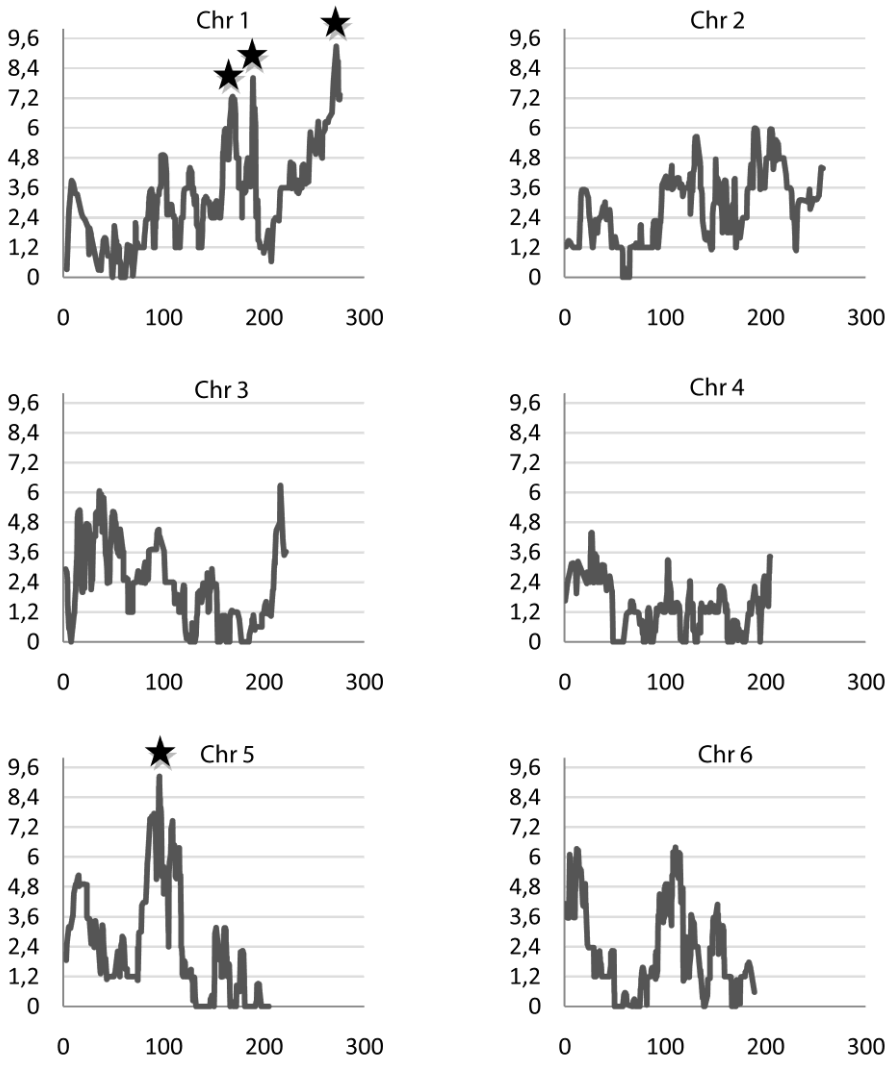
Identification of the genetic factors for unsolved WWS patients remains challenging. Nearly all patients display large regions of homozygosity as their parents are in most cases first cousins. Siblings share only a quarter of these regions on average, therefore homozygosity mapping in affected siblings can considerably reduce the number of homozygous candidate disease loci within a family. Most of the WWS families in our research cohort involve a single case of WWS (**Table 1**).

Table 1. WWS research cohort (57 families*)

Affected	single cases: 48 two siblings: 7 three siblings: 2
Diagnosis	WWS: 38 WWS-MEB-like: 19
Consanguinity	32
Mapping data	10K: 13 250K: 14

*Including only patient for which DNA is available for research; 10K, Affymetrix GeneChip® Mapping 10K 2.0 Array; 250K, Affymetrix Nsp array from the GeneChip® Human Mapping 500K Array set

The power of homozygosity mapping to resolve the genetic cause for rare autosomal recessive disorders largely depends on a homozygous candidate disease locus on the genome that is shared by affected individuals from different consanguineous families or shared by all affected kindred from a single consanguineous family with multiple affected individuals (Chapter 1). However, due to the large extent of genetic heterogeneity for WWS it is not possible to exclude homozygous candidate disease loci that are not shared between patients from different families, resulting in many regions of homozygosity. In addition, the small number of WWS families with more than one affected sibling further limits the application of homozygosity mapping for WWS. No LOD scores above 3 can be obtained in such families, which is a measure for statistical significance for a genetic localisation. Nonetheless, we performed LOD score analysis for all 27 families as a method to quantify the homozygous regions across the genome that are shared by these families. Totals of positive LOD scores for all families were calculated and plotted for each chromosome (**Figure 5**).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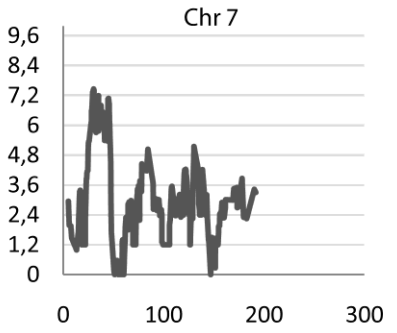
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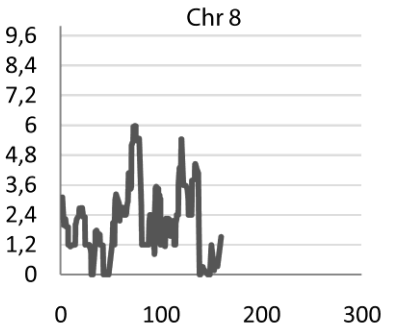
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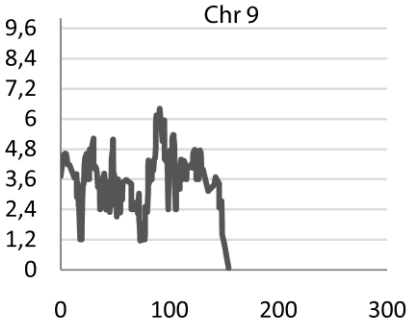
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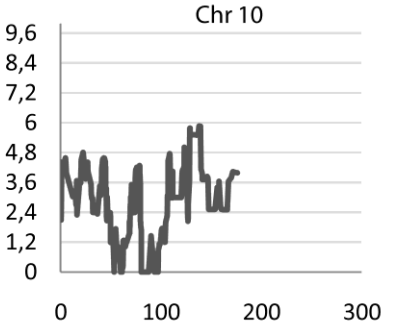
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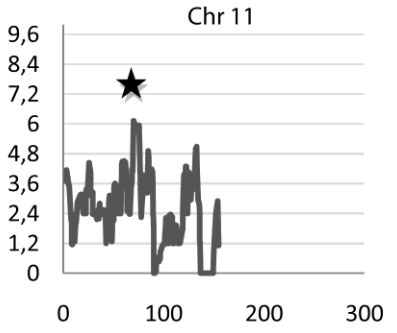
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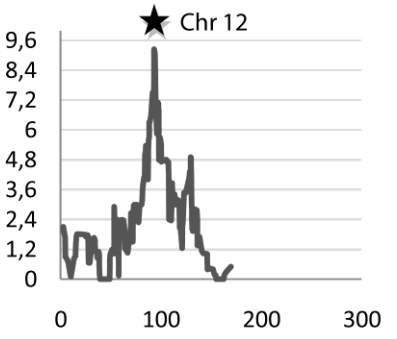
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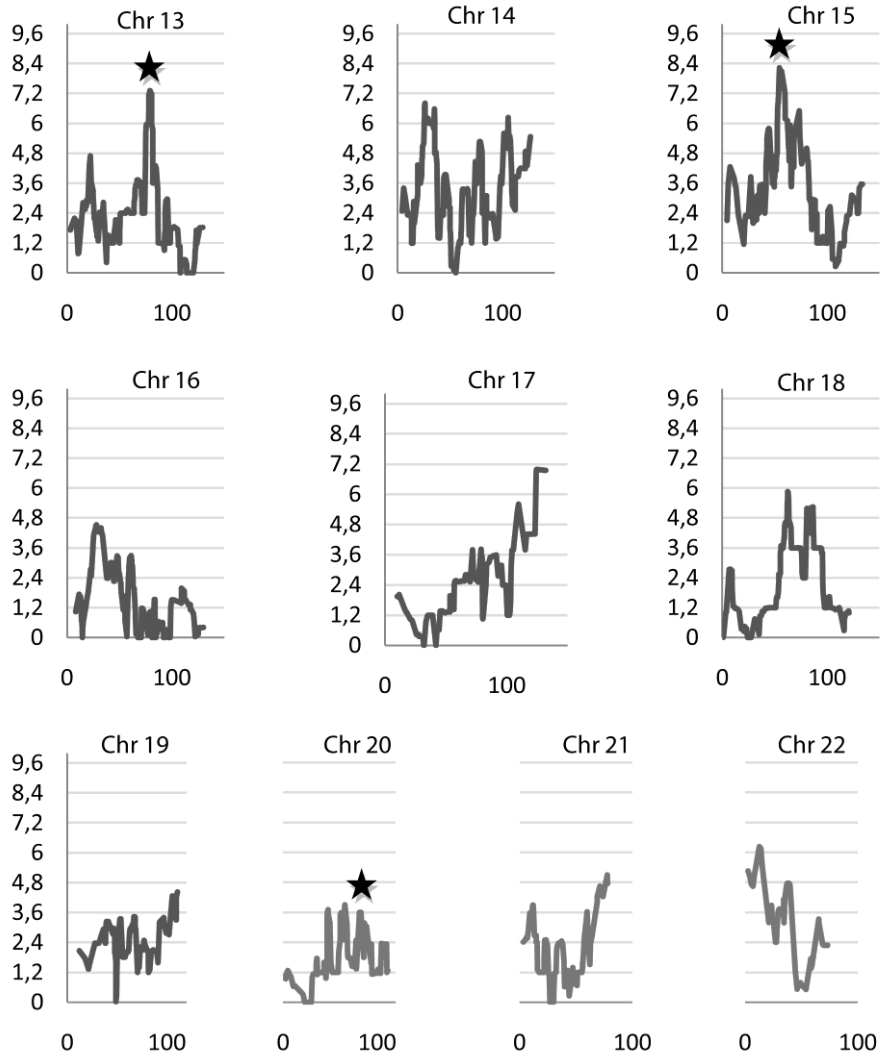

Figure 5. Overview of homozygous regions on all autosomal chromosomes for 27 WWS(-like) families. The sum of the LOD scores (vertical) is plotted for each SNP markers by genetic position (horizontal). Patients DNA samples were genotyped for SNP markers using the 10K or 250K GeneChip® Mapping arrays. Regions of homozygosity across the genome were determined by parametric multipoint LOD score analysis using the program easyLINKAGE with the Allegro algorithm (Hoffmann and Lindner, 2005). The sum of the LOD scores per marker were calculated and plotted. Black stars indicate nine regions of homozygosity as listed in **Table 3**

The majority of the patients from our cohort are born to first cousin parents. The maximum LOD score for patients from first cousin parents is 1.2 for single cases (21), 1.8 for two siblings (4), and 2.4 for three siblings (2). This maximum LOD score has been used to determine regions of homozygosity by descent. By adding up the LOD scores for all families, the maximum of the sum of LOD scores for a one-locus hypothesis is 37.2. The maximum sum of the LOD scores that we obtained is 9.3 at three chromosomal regions, 1q44, 5q14.1, and 12q21.2-21.31. These LOD scores do not reach the maximum sum of the LOD scores for a one-locus hypothesis by far, which strongly supports further genetic heterogeneity. Based on the genome-wide homozygosity data presented in **Figure 5**, I have attempted to determine the minimum number of additional WWS loci. It appeared that at least nine different non-overlapping chromosomal segments are required to cover the homozygous regions of all 27 families that were analyzed (**Table 2**). Patients from all 27 families are homozygous for at least one of these nine loci and therefore these nine regions may contain all mutations that cause WWS in these families.

Table 2. Overview of regions of homozygosity for 27 WWS(-like) families. Patients from the 27 families are homozygous for at least one of these nine loci.

						Families																								
Chr	Chr band	Sum LOD	Size (cM)	Size (bp)	UCSC genes	4	21	25	26	28	31	32	37	43	53	56	76	79	80	81	83	84	102	104	107	111	112	113	117	122	124	128
1	q44	9,4	4,7	2,5E+06	26		0,5		1,2	0,5			1,2		1,2					1,2				1,1		1,2		0,5		0,8		
1	q24.1-24.2	7,3	2,0	7,8E+05	7				1,2	1,2					1,2		1,2			0,2							1,2					1,1
1	q31.1	8,0	0,8	1,8E+06	0				0,9						1,2		1,2	1,2				1,1				1,2					1,2	
5	q14.1	9,2	1,4	1,2E+06	10		0,9										0,8	1,2	1,2					1,8			1,2	1,2		0,9		
11	q13.1-13.2	5,5	8,6	3,7E+06	87				0,4	1,2	2,5					0,8			0,1		0,3							0,2				
12	q21.2-21.31	9,4	2,0	2,0E+06	10	1,2								1,2		1,6					1,8	1,2	1,2				1,2					
13	q31.1-31.3	7,3	2,7	6,1E+06	4			2,4		1,2			0,1						1,2						1,2			1,2				
15	q21.3-22.2	8,2	6,5	3,9E+06	22	1,2			1,2	1,1					1,2				1,2				1,2						1,1			
20	q13.13-13.2	3,6	7,9	3,4E+06	14							1,8				1,8																

*Regions of homozygosity that are shared by most families (highest sum of the LOD scores) were selected. Next we selected those loci that are shared by families which are not contained in the other regions (families 25, 32, 107, 128). Grey boxes indicate the positive LOD scores for one of the 27 families for one of the selected regions. LOD scores were calculated as described in **Figure 4**.

Obviously, the most interesting candidate disease loci are those shared by most patients, represented by the highest sum of the LOD scores. However, these loci may include many genes, and mutation analysis by direct sequencing is still a laborious, costly, and time-demanding task. Prior to mutation analysis, we followed a candidate-gene-approach to select for genes predicted to functionally overlap with the products of one of more known WWS genes. The selection was based on properties of known WWS genes such as spatial and temporal expression, and on (predicted) properties of the encoded protein products, such as their possible role in

protein glycosylation. For disease loci containing a large number of genes, disease gene prioritization has been carried out by bioinformatic analysis using tools such as listed in Table 2 in Chapter 1. Yet, mutation analysis in a selection of candidate genes did not result in the identification of a new WWS gene (**Table 3**).

Table 3. WWS candidate genes analysed by direct mutations analysis.

WWS candidate locus	WWS candidate gene(s)	Mutation analysis (# of families)
3p21.31	*GMPPB*, *DAG1*	10
5p14.4-1p4.1	*CDH9*, *CDH10*, *CDH12*	3
11q13.2	*B3GNT1*	3
13q31.1-q31.3	*AF339814*, *GPC5*, *GPC6*, *NDFIP2*, microRNAs, *POU4F1*, *RBM26*, *SLITRK1*, *SLITRK5*, *SLITRK6*, *SPRY2*	4
17q25.3	*P4HB*	20
18q12.1-q12.2	*GALNT1*, *B4GALT6*	4

In our experience, homozygosity mapping in small families, for a condition with an unexpected high degree of genetic heterogeneity has met with limited success. The recent application of high-density SNP-arrays now enables the detection of small regions of homozygosity, containing few genes. The detection of such small regions and the reduction in cost for high density SNP-array analysis has enabled inclusion of individuals with remote consanguinity or without pedigree information, who nevertheless show evidence of consanguinity by an excess of homozygosity. These SNP-arrays can also detect microdeletions or duplications in homozygous intervals, which would immediately identify the causative gene. For example, the intragenic *LARGE* deletion (Chapter 4) would have been uncovered by application of the 250K SNP-array. For large regions of homozygosity containing many genes, I suggest a systematic analysis of genes for a functional link to the *O*-mannosylation pathway by a concert of bioinformatic analysis tools as is recently described for the prioritization of candidate genes for type 2 diabetes and obesity (Tiffin et al., 2006). Also filtering of genes in the homozygous regions for their presence in relevant large-scale datasets such as the secretory pathway proteome can be successful as demonstrated with the identification of the gene causing impaired glycosylation in patients with cutis laxa (Gilchrist et al., 2006; Kornak et al., 2008). Furthermore, I suggest using complementing approaches to homozygosity mapping if possible, such as genome-wide expression analysis to detect differences in expression levels resulting from mutations in new WWS genes. Another possibility is to perform RNAi targeting of genes in regions selected by homozygosity mapping in fibroblast cell lines, which are then screened for hypoglycosylation of α-dystroglycan. However, next generation

sequencing technologies, as described above, may soon enable affordable large-scale sequencing of genomic regions selected by homozygosity mapping, which obviates the need of selection of candidate genes.

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Summary / Samenvatting

Summary

The research described in this thesis was focused on the elucidation of genetic factors involved in Walker-Warburg syndrome (WWS). WWS is one of the many rare disorders caused by defects in protein glycosylation. Immunohistochemistry in muscle from WWS patients shows a defect in the *O*-linked glycosylation of α-dystroglycan. Specific *O*-mannosyl glycans are crucial for the function of dystroglycan and hypoglycosylation results in a loss of binding of extracellular ligands, such as laminin-α2. Consistent with other disorders showing an autosomal recessive pattern of inheritance, the incidence of WWS is higher in populations with a high rate of consanguineous unions. This phenomenon was exploited to identify candidate loci by a specific method of linkage analysis, homozygosity mapping (**Chapter 1**).

Prior to the work described in this thesis, autosomal recessive mutations were reported in three genes, *FKRP*, *FKTN*, and *POMT1*, in approximately one-fifth of the WWS patients in our research cohort (reviewed in **Chapter 2**). We performed homozygosity mapping in WWS patients, from consanguineous pedigrees and without mutations in these three genes. Next we performed mutation analysis for genes within the critical regions, that code for proteins with a possible role in *O*-linked glycosylation of α–dystroglycan. This resulted in the identification of mutations in two new WWS genes, *POMT2*, and *LARGE*. We identified *POMT2* mutations in three of six families showing homozygosity at the *POMT2* locus (**Chapter 3**). Immunohistochemistry in muscle showed markedly reduced levels of glycosylated α-dystroglycan, which is consistent with the requirement of an active enzyme complex of POMT1 and POMT2 to obtain *O*-mannosyl transferase activity. Despite the common structural and functional characteristics of POMT1 and POMT2, we and others detected a lower mutation frequency for *POMT2* in WWS and related disorders.

A spontaneous deletion of *Large* in mice is associated with a severe neuromuscular phenotype that resembles WWS-like disorders. We identified a 63-kb intragenic deletion in the *LARGE* gene in one of the six WWS families homozygous at the *LARGE* locus by copy number analysis and direct sequencing, thereby describing the fifth WWS gene (**Chapter 4**).

Mutations in the six genes that encode for putative or demonstrated glycosyltransferases, *FKRP*, *FKTN*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2*, give rise to a range of dystroglycanopathies with a much wider spectrum of clinical manifestations than previously described (**Chapter 7**). Within the group of dystroglycanopathies, phenotypic diversity not only occurs for different mutated genes but also for different mutations within each of the genes. Consequently, it is currently not possible to predict the involvement of a specific gene, solely on the basis of the phenotype. A correlation between the predicted effect of a mutation on the protein function, and the resulting clinical phenotype, also referred to as a genotype-phenotype correlation, can however be observed for some mutations in specific genes within the *O*-linked glycosylation pathway. This is most evident for WWS where nearly all mutations are predicted to completely abolish protein function. We identified *POMT1* null mutations in WWS patients and compound heterozygous *POMT1* mutations in a group of patients with a relatively mild condition of congenital muscular dystrophy and mental retardation (**Chapter 5**). The majority of known *FKRP* mutations result in amino acid changes causing a limb-girdle muscular dystrophy or a relatively mild congenital muscular dystrophy. However, we identified a homozygous *FKRP* start codon mutation in two WWS siblings, suggesting that null mutations in *FKRP* result in the severe WWS (**Chapter 6**). The mutations we identified in WWS patients in *POMT2* (**Chapter 3**) and *LARGE* (**Chapter 4**) are likely to abolish the corresponding protein functions. Likewise, truncating mutations within the *FKTN* gene are associated with WWS. Less phenotypic diversity is seen for *POMGnT1* mutations, where most mutations give rise to muscle-eye-brain disease.

We have described the genetic cause for WWS in approximately one-third of the patients in our research cohort by the identification of mutations in *FKTN*, *FKRP*, *LARGE*, *POMT1*, and *POMT2*. We predict the existence of at least nine other WWS loci based on our genome-wide homozygosity data from 27 additional WWS families (**Chapter 7**). This suggests an unexpectedly high degree of genetic heterogeneity for this rare but clinically recognizable syndrome, and underscores the complexity of the glycosylation machinery for α-dystroglycan. Finding additional causative genes for WWS will shed more light on processes involved in *O*-linked glycosylation of α-dystroglycan and may provide clues for new candidate genes for WWS. The nine additional loci selected by homozygosity mapping in 27 WWS families contain at least 180 genes. Candidate disease gene prioritization will be necessary to select genes for mutation analysis by direct sequencing in this valuable cohort. Next generation high-throughput sequencing technologies may soon enable sequencing of the complete homozygous genomic regions in these patients, which will obviate the

often difficult task of candidate gene selection. Given the presence of genetic heterogeneity, high-throughput sequencing will also be desirable for DNA-diagnostic analysis of WWS, when all underlying genes have been identified.

Samenvatting

Het in dit proefschrift beschreven onderzoek was gericht op het ontrafelen van de genetische factoren die het Walker-Warburg syndroom (WWS) kunnen veroorzaken. WWS is één van de vele zeldzame aandoeningen die veroorzaakt worden door defecten in de eiwitglycosylering. Immunohistochemie in spierweefsel van WWS patiënten toont een defect in de *O*-gebonden glycosylering van het eiwit α–dystroglycaan. Specifieke *O*-mannosyl glycanen zijn cruciaal voor de functie van dit eiwit en hypoglycosylering resulteert in een verlies van binding van extracellulaire liganden, zoals laminine α2. WWS patiënten worden gekenmerkt door ernstige aangeboren hersen- en oogafwijkingen en spierdystrofie. De incidentie van WWS is, overeenkomstig met andere autosomaal recessieve aandoeningen, hoger in bevolkingsgroepen met een hoog aantal consanguïne verbintenissen. Dit fenomeen is benut om kandidaatloci te identificeren middels een specifieke methode van koppelingsonderzoek, "homozygosity mapping" (**Hoofdstuk 1**).

Voorafgaand aan het in dit proefschrift beschreven onderzoek werden bij ongeveer een vijfde van de WWS patiënten uit ons onderzoekscohort autosomaal recessieve mutaties beschreven in drie genen, *FKRP*, *FKTN*, en *POMT1* (**Hoofdstuk 2**). Voor WWS patiënten met consanguïne ouders en zonder mutaties in deze drie genen werd homozygosity mapping uitgevoerd. Vervolgens werd mutatieanalyse uitgevoerd voor genen uit homozygote regio's die coderen voor eiwitten die mogelijk een rol spelen in de *O*-gebonden glycosylering van α–dystroglycaan. Dit heeft geresulteerd in de identificatie van twee nieuwe WWS genen, *POMT2* en *LARGE*. *POMT2* mutaties werden geïdentificeerd in drie van de zes families die homozygoot waren voor het *POMT2* locus (**Hoofdstuk 3**). In spierweefsel werden duidelijk gereduceerde niveaus van geglycosyleerd α–dystroglycaan aangetoond met immunohistochemie, wat overeenkomt met een vereist actief enzym complex van POMT1 en POMT2 voor *O*-mannosyl transferase activiteit. Hoewel POMT1 en POMT2 structureel en functioneel veel overeenkomsten vertonen, hebben wij en anderen een lagere mutatie frequentie voor *POMT2* in WWS en gerelateerde ziekten gevonden.

Een *de novo* deletie van *Large* in muizen veroorzaakt een ernstig neuromusculair fenotype dat vergelijkbaar is met WWS-achtige ziekten. In één van de zes WWS families die homozygoot waren voor het *LARGE* locus hebben we een 63-kb intragene deletie in het *LARGE* gen gevonden door DNA kopieaantal analyse en DNA-sequentie analyse. Door dit resultaat zijn thans vijf WWS genen opgehelderd (**Hoofdstuk 4**).

Mutaties in zes genen, die coderen voor veronderstelde of bewezen glycosyltransferases, *FRKP*, *FKTN*, *LARGE*, *POMGnT1*, *POMT1* en *POMT2*, resulteren in een reeks van dystroglycaanaandoeningen met een veel breder spectrum van klinische verschijnselen dan voorheen was beschreven (**Hoofdstuk 7**). Fenotypische diversiteit binnen de groep van dystroglycaan-aandoeningen is niet alleen het resultaat van verschillende gemuteerde genen, maar ook van verschillende mutaties in elk van de genen. Als gevolg hiervan is het momenteel niet mogelijk om de betrokkenheid van een bepaald gen uitsluitend aan de hand van het fenotype te voorspellen. Voor sommige mutaties in specifieke genen die betrokken zijn bij de *O*-gebonden glycosyleringsroute zien we echter wel een correlatie tussen het voorspelde effect van een mutatie op eiwitfunctie en het resulterende fenotype. Dit wordt ook wel een genotype-fenotype correlatie genoemd. Deze correlatie is het meest duidelijk voor WWS, waarbij voor de meeste mutaties wordt voorspeld dat ze de eiwitfunctie tenietdoen. In *POMT1* hebben we met name homozygote nul mutaties geïdentificeerd in WWS patiënten, terwijl heterozygote *POMT1* mutaties werden gevonden in een groep patiënten met een relatief milde aandoening van aangeboren spierdystrofie en mentale retardatie (**Hoofdstuk 5**). De meerderheid van de bekende *FKRP* mutaties resulteert in aminozuurveranderingen en veroorzaken limb-girdle spierdystrofie, een spieraandoening die zich pas op een latere leeftijd manifesteert, of relatief milde vormen van aangeboren spierdystrofie. Wij hebben echter een homozygote *FKRP* startcodon mutatie gevonden in twee WWS patiënten uit één familie, wat suggereert dat nul mutaties in *FKRP* resulteren in het ernstige WWS (**Hoofdstuk 6**). De in WWS patiënten geïdentificeerde mutaties in *POMT2* (**Hoofdstuk 3**) en *LARGE* (**Hoofdstuk 4**) zijn hoogstwaarschijnlijk ook destructief voor de corresponderende eiwitfuncties. Hetzelfde geldt voor stopmutaties in het *FKTN* gen die geassocieerd zijn met WWS. Voor *POMGnT1* is een genotype-fenotype correlatie minder duidelijk en zijn de meeste mutaties geassocieerd met muscle-eye-brain disease.

Door de identificatie van mutaties in *FKTN*, *FKRP*, *POMT1* en *POMT2* hebben we voor ongeveer een derde van alle patiënten uit ons onderzoekscohort de genetische oorzaak van WWS beschreven. Op basis van de analyse van onze genoomwijde homozygosity mapping data van 27 additionele WWS families voorspellen we het

bestaan van tenminste negen andere WWS loci (**Hoofdstuk 7**). Dit suggereert een onverwacht hoge mate van genetische heterogeniteit voor dit zeldzame maar klinisch herkenbare syndroom, en onderstreept de complexiteit van het glycosyleringsmechanisme voor α-dystroglycaan. Het vinden van nieuwe oorzakelijke WWS genen geeft mogelijk inzicht in tot nu toe onbekende processen betrokken bij *O*-gebonden glycosylering van α-dystroglycaan. De negen loci, geselecteerd met behulp van homozygosity mapping in 27 WWS families, bevatten minstens 180 genen. Het prioriteren van kandidaatgenen zal noodzakelijk zijn voor de selectie van genen voor DNA-sequentie analyse. Nieuwe high-throughput sequentietechnologieën maken het binnenkort wellicht mogelijk om complete homozygote gebieden in deze patiënten te sequencen wat de moeilijke taak van kandidaatgenselectie zal ondervangen. Gegeven de genetische heterogeniteit voor WWS is high-throughput sequencen ook wenselijk voor WWS DNA-diagnostiek analyse wanneer alle onderliggende genen zijn geïdentificeerd.

Curriculum vitae

Jeroen van Reeuwijk werd geboren op 29 maart 1976 te Zoetermeer. In 1993 behaalde hij zijn diploma voor het Hoger Algemeen Voortgezet Onderwijs aan de Goudse Scholen Gemeenschap. Vervolgens volgde hij Hoger Laboratorium Onderwijs aan de Hogeschool van Utrecht met als studierichting Medische Biotechnologie. Een afstudeerstage van negen maanden verrichtte hij in het laboratorium van prof. dr. Heffron aan de Oregon Health Sciences University te Portland (OR, VS). Onder supervisie van dr. Brian Ahmer werkte hij daar aan de identificatie en karakterisatie van Salmonella genen die betrokken zijn bij infecties veroorzaakt door deze bacterie. Na de opleiding in 1997 voltooid te hebben is hij teruggekeerd naar de Verenigde Staten waar hij het Salmonella onderzoek nog een jaar heeft voortgezet. Eenmaal terug in Nederland heeft hij drie jaar gewerkt als onderzoeksassistent bij Numico Research (nu Danone) te Wageningen. In september 2001 startte hij aan de Wageningen Universiteit een masterstudie biotechnologie, met als specialisatie bioinformatica. Onder supervisie van dr. Peter Groenen werkte hij acht maanden aan zijn afstudeerstage binnen de bioinformatica-groep van Organon (nu Schering-Plough) te Oss. Tijdens deze stage bestudeerde hij orthologie en clustermethoden om clusters van gerelateerde eiwitten te definiëren binnen een uitgebreide dataset van eiwitsequentievergelijkingen. Na zijn afstuderen in maart 2003 was hij van juni 2003 tot oktober 2007 aangesteld als junior onderzoeker binnen de afdeling Antropogenetica van het UMC St Radboud te Nijmegen. Onder supervisie van prof. dr. Han Brunner en dr. Hans van Bokhoven verrichtte hij het in dit proefschrift beschreven onderzoek naar de erfelijke factoren die een rol spelen bij het veroorzaken van het Walker-Warburg syndroom. In deze periode werkte hij anderhalf jaar binnen de comparative genomics groep van het Centre for Molecular and Biomolecular Informatics onder supervisie van prof. dr. Martijn Huynen. Hier deed hij onderzoek naar een methode om, middels evolutionair geconserveerde co-expressie data, kandidaatziektegenen te prioriteren. Sinds januari 2008 is hij werkzaam als postdoc in de groep van dr. Ronald Roepman op de afdeling Antropogenetica van het UMC St Radboud te Nijmegen. Hier verricht hij onderzoek naar het interactome van eiwitten betrokken bij cilia-gerelateerde vormen van erfelijke blindheid.

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